An Investigation of Corticotropin-like and Opiate-like Substances in Tissues of Different Vertebrates

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ABBREVIATIONS

ACTH	: Adrenocorticotropin
AAP	: Acid acetone powder
AP	: Acetone powder
B-P	: Combined brain and pituitary fraction
BSA	: Bovine serum albumin
BT	: Bovine testes
CM-cellulose	: Carboxylmethyl-cellulose
DADLE	: H-D-ala^{2}-D-leu^{5}-enkephalin
EB	: Grouper (*Epinephelus akaara*) brain
EE	: Eel pancreas
Fl	: Flounder pituitary
GB	: Gerbil heart
GP	: Guinea pig heart
HH	: Hamster heart
[^{125}I]-β-endorphin	: (3-[^{125}I]iodotyrosyl^{27})β-endorphin (human)
IH	: Interrenal heart
IT	: Interrenal tail
KRB	: Krebs-Ringer bicarbonate buffer
LEK	: Leucine-enkephalin
LPH	: Lipotropin
MCH	: Melanin concentrating hormone
MSH	: Melanotropin
OP	: Ovine pancreas
PB	: Pigeon brain
POMC	: Proopiomelanocortin
RH	: Rat heart
SB	: Snake brain
SP	: Snake pituitary

TH : Turtle heart

TI : Turtle intestine

ABSTRACT

Eight different tissues from thirteen different species (six of them being mammalian species) have been tested for ACTH-like and opiate-like activities. The tissues and species studied included the heart tissues of rat, hamster, guinea pig, gerbil and turtle; bovine testis, ovine and eel pancreas, brains of pigeon, snake and grouper, snake and flounder pituitaries, and various tissues of flounder and lamprey. Opiate-like and corticotropin-like activities were extracted from the tissues with a mixture of acetone, HCl and water and then subjected to chromatographic purification procedures such as gel filtration and/or ion-exchange chromatography. Opioid activity in the tissues was monitored by testing the competition with [^{3}H]-D-ala^{2}-D-leu^{5}-enkephalin (DADLE) for binding to rat brain synaptosomes. ß-Endorphin-like immunoreactivity was monitored by the displacement of [^{125}I]ß-endorphin from binding to a specific antiserum against human ß-endorphin. Corticotropin-like activity was monitored by their stimulatory effects on the production of corticosterone from dispersed rat adrenal decapsular cells.

ß-Endorphin activity was detected in most of the tissues under studied. Both opiate-like and corticotropin-like activities were demonstrated in most of the tissues studies except that opioid activity was not found in the pigeon brain; and that corticotropin-like activity was not detected in the turtle heart, eel pancreas and turtle intestine. Studies on various tissues of the flounder revealed the presence of

corticotropin-like activity in the combined brain & pituitary extract and interrenal tissues but not in the heart, intestine gonad, or liver while opiate activity was found in most tissues the except gonad. ß-Endorphin-like immunoreactivity was found in the combined brain and pituitary and the interrenal tissues of flounder but not in any tissues of lamprey. In general, opioid and corticotropin-like activities in the tissues were retarded on Sephadex G-25 and adsorbed on CM-cellulose indicating that they were basic in character and possessed a molecular weight of less than 5,000 like authentic corticotropin and opioids from the mammalian pituitary. However, a portion of the activities in some tissues was unretarded on Sephadex G-25 and implying a molecular weight larger than 5,000.

Studies on various tissues of lamprey revealed the presence of corticotropin-like activity in the brain and liver but not in the pituitary, heart, intestine, ovary or testis while opiate activity was found in the liver, heart, gut, brain and pituitary but not in the gonad. ß-Endorphin-like immunoreactivity was not found in any of the lamprey tissues studied. The potencies of the activities found in the assays depend on the tissues, the species studied and the purification method applied.

CONTENT

INTRODUCTION

Chapter 1. INTRODUCTION

1.1 Relationship between adrenocorticotropin and opiates

1.1.1 POMC is the common precursor of ACTH and opiates

Adrenocorticotropin (ACTH) is an important peptide hormone secreted from the anterior pituitary of mammals. It has been demonstrated that this peptide is derived from a poly-protein called 'proopiomelanocortin' (POMC) in the pituitary (Hope and Lowry, 1981). POMC is now found to be also the precursor of many other pituitary hormones including α-MSH, β-MSH, β-LPH, γ-LPH, and β-endorphin (Chretien and Seidah, 1984).

When first discovered, β-lipotropin (β-LPH) (Li and Chung, 1976a), one of the derivatives from POMC, was found to contain the sequence of methionine enkephalin, the first discovered endogenous opioid peptide. β-LPH was therefore suspected to be an opioid precursor. Further characterization of this peptide led to the discovery of a fragment that possesses this particular sequence and opiate activity (Cox et al, 1976; Graf et al, 1976). This fragment was found to have 31 amino acids and possess little lipolytic activity (Li and Chung, 1976b). It is in fact a new endogenous opioid derived from POMC and is now called β-endorphin.

1.1.2 ACTH and opiates work together in the body

Some aspects about the actions of ACTH and endogenous opioid peptides are discussed here to show the relationship among these regulatory peptides in the body.

1.1.2.1 Adrenal cortex is a target tissue of POMC-derived peptides other than ACTH

Adrenal cortex is the conventional target tissue of ACTH. It was found that concentrations of β-endorphin and β-LPH increase progressively from prepuberty to puberty while that of ACTH remains rather constant (Genazzani et al, 1983). In view of the fact that the N-terminal fragments of POMC possess mitogenic activity (Estivariz et al, 1981) in the rat adrenal under physiological conditions, it was then proposed that POMC-related peptides other than ACTH regulate adrenal cell growth whereas the steroidogenic cascade remains under the control of ACTH (Genazzni et al, 1983). This proposal was supported by the recent discovery that pro-γ-MSH, a peptide derived from the N-terminal of POMC, is a trophic factor of adrenal cortex (Lowry et al, 1984-85).

1.1.2.2 Endogenous opioid peptides regulate the level of ACTH in the body

β-Endorphin was found to suppress ACTH and cortisol (a cortical steroid hormone secreted by the adrenal under the stimulation of ACTH in human beings) levels in normal human subjects (Taylor et al, 1983), indicating an indirect regulation of cortisol output through a feedback mechanism on

the POMC synthesizing and/or processing system. Furthermore, direct evidence of the inhibitory effects of endogenous opioid peptides on the release of corticotropin releasing factor (CRF), the stimulatory factor of ACTH in hypothalamus, was found by using rat hypothalamus (Yajima et al, 1986). However, a direct influence of ß-endorphin on the cortisol response to ACTH was also found (Beyer et al, 1986).

1.1.2.3 ACTH also affects the opiate system in the body

Analgesic effects of ACTH with a potency equivalent to morphine have been reported (Walker et al, 1980). However, it is also thought that ACTH may have a physiological role as an endogenous contra-opioid agonist (Hendrie, 1985) as it reverses the actions of opiates (Hendrie, 1986). Although it was suggested that POMC-derived peptides other than ß-endorphin play a physiological role against the potentially long-lasting effects of ß-endorphin-induced analgesia (Akil et al, 1986), the controversal problem remains to be uncovered in the future.

1.1.2.4 Stress stimulates both ACTH and endogenous opioid peptides

ACTH is long known as the hormone that copes with stress in the body (Jones et al, 1981). Recently, endogenous opioid peptides (Terman et al, 1984) have also been found to be stimulated by stress and play important roles in stress responses. Furthermore, it is now well-known that POMC processing and production as well as the release of its

products - ACTH and ß-endorphin, in both anterior and intermediate lobe of pituitary, are induced by stress (Akil et al, 1986). This results in the elevation of the level of these two important hormones in the body.

1.2 Adrenocorticotropin (ACTH)

Adrenocorticotropin (ACTH) secreted from the pituitary gland of mammals stimulates the adrenal cortex to produce corticosteroids. Although it is a peptide with 39 amino acids, its ability to stimulate adrenocortical tissue resides only in the first 24 amino acids at the N-terminal of the molecule (Schwyzer et al, 1971).

1.2.1 ACTH works in various tissues of the body

Although ACTH was traditionally regarded as a hormone secreted from the pituitary gland, adrenocorticotropic activities were recently found in many extra-pituitary tissues (Saito et al, 1983). Tissues including the pituitary, brain, placenta, lung, spleen, adrenal, gonads, and pancreas (LeRoith et al, 1986) have already been proposed to be possible sites of ACTH production. The presence of ACTH in mouse spleens (Smith et al, 1982) and human leukocytes (Meyer et al, 1987) suggests a possible direct action of the hormone on the immune system. Furthermore, its precursor, POMC, and even the mRNA of POMC, were found in tissues such as the adrenal medulla, thyroid, thymus, lung, duodenum, hypothalamus (Jingami et al, 1984; Steenbergh et al, 1984) and testes (Chen et al, 1984; Pintar et al, 1984), indicating a possible local synthesizing system of ACTH in these extra-pituitary tissues. Besides stimulating the adrenal cortex, ACTH also affects the

functions of other tissues (Acker et al, 1984; Armario et al, 1986). However, the actual physiological roles of ACTH in the various extra-pituitary tissues remains to be elucidated.

1.2.2 ACTH is found in various organisms

The amino acid sequence of ACTH is highly conserved among the various mammalian species studied. Even in the C-terminal which was originally thought to be variable from one mammalian species to another, only two positions, 31 and 33, show heterology among porcine, human, ox, and sheep (Lowry et al, 1977). Furthermore, ACTH isolated from dogfish was found to have a structure similar to that of the mammals (Lowry et al, 1974), indicating that the peptide may have a role in lower vertebrates. Recent studies even indicate its existence in some multicellular invertebrates (Kaloustian, 1986; LeRoith et al, 1986) and unicellular organisms (LeRoith et al, 1982). These findings suggest that ACTH may play a role in many lower animals.

1.3 Endogenous opioid peptides

The history of endogenous opiate peptides started when the two pentapeptides, methionine- and leucine-enkephalins, referred to as naloxone reversible activity, were discovered (Hughes, 1975). In the following year, a larger endogenous opiate, ß-endorphin, was discovered (Graf et al, 1976; Cox et al, 1976). Up until now, three opioid peptide gene families have been discovered. They are the POMC family giving ß-endorphin and other three endogenous opioid peptides; the proenkephalin family giving met- and leu-enkephalins and other thirteen; and the prodynorphin family giving eight more dynorphin-related opioid peptieds (Kitchen, 1985).

1.3.1 Opioid receptors

Opioid peptides exert their various functions through three different types of opioid receptors: μ, δ, and k. Different families of opioid peptides show different preferences to different receptors (Hughes J. 1984). However, it has been suggested that there exist at least four, or even five, types of opioid receptors either with separate or with common genetic entities (Yaksh and Noueihed, 1985). If the subtypes of μ-receptor (Pasternak and Wood, 1986) and k-receptor (Sanchez-Blazquez et al, 1984) are also counted, the total number of opioid receptors will be pushed up to seven. The biochemical natures of these receptors are still not fully

known although a k-receptor with a molecular weight of 400000 (Itzhak et al, 1984) and a μ-receptor with a molecular weight of 58000 (Cho et al, 1986) were claimed to have been purified.

1.3.2 Opioid peptides work in various tissues of the body

1.3.2.1 Neuronal functions of opioid peptides

The neuronal functions of opioid peptides are rather complicated. The function of opioid peptides as neuromodulators or neurotransmitters in the neuronal communication has been proposed (Zieglgansberger, 1984). Besides their analgesic effects, the actions of endogenous opioid peptides were found to be correlated to other neurotransmitters such as acetylcholine, catecholamines, serotonin, and γ-amino-butyric acid (Oliverio et al, 1984). Behavioral studies have indicated that opioids also play a role in sleep, tolerance, learning, and memory (Oliverio et al, 1984). Opioids also affect the functions of peripherial tissues such as motor, cardiovascular, gastrointestinal, and bladder functions through the spinal cord (Yaksh and Noueihed, 1985).

1.3.2.2 Physiological functions of opioid peptides

The physiological functions of opioids are in fact very complicated as reflected in the multiplicity and heterogeneity of opioid peptides and receptor systems. As endogenous opioid peptides have a widespread but differential distribution in the body, a term, "endocrine-like opioid systems" (Millan and Herz, 1985), was introduced to discribe

their various physiological functions. They not only affect the secretion of all the pituitary hormones through the hypothalamic-pituitary axis (Millan and Herz, 1985), but also many other peripheral organs such as the adrenal gland, pancreas (Millan and Herz, 1985), heart (Akil et al, 1984) and even the immune system (Husband et al, 1987). However, the stimulatory effects of β-endorphin on human lymphocyte natural killing function was found to be not so potent as its non-opioid fragments (Kay et al, 1987). The detailed discussion of the physiological functions of opioid peptides in various peripheral tissues will be discussed in subsequent chapters.

1.3.3 Opioid peptides are found in various organisms

As in the case of ACTH, opiate peptides have a wide-spread distribution in different organisms. Recent findings have also reported the existence of β-endorphin (LeRoith et al, 1982; Pestarino, 1985), the opiate derived from POMC which is also the precursor of ACTH, and enkephalin (Georges and Dubois, 1984), the opiate derived from pro-enkephalin, in inverterbates. Furthermore, opioid peptides were also found in higher plants such as wheat (Zioudrov, et al, 1979). The three opioid precursors and their mRNAs were also discovered in different tissues of some species (Schwartz and Costa, 1986), implying the possibility of their synthesis in peripherial tissues.

1.4 The new endogenous opiates : non-peptide opiates

The discoveries of morphine in bovine brain and adrenal gland (Goldstein et al, 1985) and toad skin (Oka et al, 1985) have aroused an interest in examining the possibility of existence of endogenous nonpeptide opioids. Recently, it was also found in human cerebrospinal fluid of normal subjects at a concentration comparable to that of endogenous opioid peptides (Cardinale et al, 1987). Administration of intermediates of morphine biosynthesis isolated from plants to rat tissues resulted in an increase in morphine level (Donnerer et al, 1986) implying the presence of an endogenous synthetic system for morphine in animals. Perhaps these findings may change the whole view on the endogenous opioid system in the future.

1.5 Strategy of study

In this thesis, the existence of corticotropin-like peptides was examined by a steroidogenesis assay using isolated rat adrenal cortical cells. An opiate receptor binding assay using fresh rat brain membrane was applied for the detection of the opiate receptor binding activity. Among the major endogenous opioid peptides, β-endorphin is the only one derived from POMC. Thus a β-endorphin RIA was applied for the detection of the presence of this particular opioid peptide as a further exploration of the nature of the material that possesses opiate receptor binding activity. As two different binding assays, opiate receptor binding assay and β-endorphin RIA, were applied in this investigation, most of the results from the opiate receptor binding assay will be presented in tables whereas those of β-endorphin RIA will be in graphs to prevent any possible confusion of the results derived from the two assay systems.

The samples tested were obtained from vertebrates in great diversity involving mammals, birds, reptiles, fishes and finally the cyclostome, the most primitive vertebrates in the modern world. Then the results in this thesis will indicate the existence of ACTH and endogenous opiates through out the Phylum Chordata or more strictly, Subphylum Vertebrata. As β-endorphin and ACTH are POMC-derived peptides, the co-existence of opiate (and perhaps β-endorphin) and ACTH activities in the same tissue may suggest a possible existence of POMC and/or

its processing system in these tissues. However, the chance of the opiate-like material found in the opiate binding assay to be the smaller opiates such as enkephalin, dynorphin, or even morphine-like nonpeptides cannot be ruled out since the more complicated the regulatory molecule, the lower the opportunity we could find it in simpler vertebrates.

In later chapters, the tissues studied and the methods of assay will be discussed first as a reference (Chapters 2 & 3). Subsequent chapters (Chapters 4 - 8) will deal with the results obtained from various vertebrates in an order tracing back from the most complicated mammalian tissues down the phylogenetic tree of vertebrates (Young, 1983a) to the simplest vertebrate, each with a discussion on the findings in that particular tissue. After that, the results will be summarized in chapter 9 with a tissue-oriented approach for comparing the same tissue from different vertebrates. Finally, the thesis will end up in a general discussion on the overall results, implications and perspectives in chapter 10.

MATERIALS

Chapter 2 MATERIALS

2.1 Animals

Male Sprague-Dawley rats, weighing 180 gm - 200 gm, originated from Charles River Laboratory (Japan) and maintained on Purina Chow and water, were used for opiate receptor binding assay.

Male Sprague-Dawley rats, weighing 400 gm - 450 gm, originated from Charles River Laboratory (Japan) and maintained on Purina Chow and water, were used for hormone-induced steroidogenesis assay.

2.2 Materials

All the reagents and chemicals used were of analytical grade or the best quality available. The suppliers of the chemicals used are listed in table 2-1.

Table 2-1. Suppliers of materials

Materials	Supplier
Acetic acid	Merck
Adrenocorticotropin (ACTH) (Porcine)	Sigma
Bacitracin	Sigma
Bovine serum albumin (BSA)	Sigma
Bovine testes acetone powder	Sigma
Collagenase (Type II)	Sigma
Corticosterone	Sigma
1,2,6,7-[^{3}H]-corticosterone	Amersham
Corticosterone antiserum	Miles
CM-cellulose	Sigma
D-ala -D-leu -(tyrosyl-3,5-[^{3}H])enkephalin [DADLE]	Amersham
Dextran T-70	Pharmacia Fine Chemical
Dynorphin (1-13)	Sigma
Eel pancreas acetone powder	Sigma
α-Endorphin	Gift from Dr. C. H. Li
β-endorphin antiserum (rabbit)	Amersham
Flounder tissues	Gift from Dr. D. R. Idler
D-glucose	BDH
(3-[^{125}]iodotyrosyl^{27})-β-Endorphin	Amersham
Lamprey tissues	Gift from Dr. G. Wright
Leucine-enkephalin	Sigma
Lima bean trypsin inhibitor	Sigma
Methionine-enkephalin (Met-enkephalin	Sigma
α-Melanotropin (α-MSH)	Sigma
β-Melanotropin (β-MSH)	Sigma

Materials	Supplier
β-Melanotropin (β-MSH)	Sigma
Norit A (charcoal)	Serva
Ovine pancreas acetone powder	Sigma
Pigeon brain acetone powder	Sigma
POPOP (2,2'-phenylen-bis(5-phenyloxazole))	Merck
PPO (2,5-diphenyloxazole)	Sigma
Sephadex G-10	Sigma
Sephadex G-25	Sigma
Snake brain & pituitary	Local snake shop in H. K.
Sucrose	Merck
Thimerosal	Sigma
Trizma base [Tris(hydroxymethyl)amino-methane]	Sigma
Triton X-100 (Octylphenoxy polyethoxy-ethanol)	Sigma
Trypsin	Worthington
Turtle heart acetone powder	Sigma
Turtle intestine acetone powder	Sigma
Vasoactive intestinal peptide (VIP)	Gift from Dr. SW. Said

ASSAY SYSTEMS

Chapter 3 ASSAY SYSTEMS

3.1 Hormone-induced steroidogenesis

3.1.1 Principle of bioassay of steroidogenic activity

The adrenal cortex is divided into three zones : zona glomerulosa, zona fasciculata, and zona reticularis. The outer zona glomerulosa is chiefly concerned with biosynthesis of the mineralocorticoids whereas the two inner zones are stimulated by adrenocorticotropin (ACTH) to secrete glucocorticoids. In the rat, the glucocorticoid secreted by the two zones is corticosterone instead of cotisol which is secreted by the human adrenal cortex.

In our assay system, the steroidogenic activities of unknown samples were measured in terms of the amount of corticosterone generated after the sample had been incubated with dispersed rat adrenal cortical cells containing the two glucocorticoid producing zones.

3.1.2 Method of steroidogenesis assay

3.1.2.1 Isolation of rat adrenal decapsular cells

Adrenal cells were prepared by the method of Li (Li et al, 1982) with minor modifications. Male Sprague-Dawley rats weighing 400-450 gm (about thirteen weeks old) were sacrificed

by cerivical dislocation. Adrenal glands were carefully removed and trimmed free of fat. The capsules consisting mainly of glomerulosa cells were separated from the inner zones made up mainly of fasciculata and reticularis cells.

The decapsulated glands were minced and suspended in freshly prepared Krebs-Ringer buffer (1 ml/gland) containing collagenase (Sigma type II, 3 mg/ml), D-glucose (2 mg/ml) and bovine serum albumin (Sigma fraction V, 4 mg/ml) in polypropylene culture tubes. The tubes were then saturated with 95% O_2 - 5% CO_2 and incubated in a Dubnoff metabolic shaker at 37 °C with gentle shaking (65-75 cycles/min) for 1 hour.

The tubes were then allowed to stand upright until the tissue had settled. The incubation medium , which was the fresh Krebs-Ringer bicarbonate buffer containing 0.4% bovine serum albumin, 0.2% D-glucose and 0.1% lima bean trypsin inhibitor (Sigma), was then added (0.5 ml/adrenal) and the adrenal cells were dislodged from the tissue by repeatedly drawing the suspension into and out of a Pasteur pipette. The pieces of tissue were then allowed to settle and the supernatant containing the dispersed cells was collected and filtered through four layers of cheesecloth. The procedure was repeated at least twice to ensure completeness of cell dispersal. The cell suspension was centrifuged at 50 g for 5 minutes at room temperature in a MSE GF-8 centrifuge. The supernatant was removed by aspiration and the cells were resuspended in the same volume of incubation medium, washed twice and then the cell concentration was adjusted to about 300,000 / ml.

3.1.2.2 Incubation of dispersed adrenal decapsular cells with test samples

Aliquots of the cell suspension (180 ul contain about 54,000 cells) were transferred to polypropylene culture tubes containing a solution of the sample to be assayed so as to make up a final assay volume of 200 ul. The tubes were then incubated at 37 °C for 2 hours under an atmosphere of 95% O_2 : 5% CO_2 with moderate shaking in a Dubnoff metabolic water both. At the end of the incubation, the tubes were frozen in -20 °C freezer until determination of corticosterone concentration by radioimmunoassay.

3.1.2.3 Corticosterone RIA

Corticosterone was measured by radioimmunoassay using a rabbit anti-corticosterone serum. Corticosterone standards and samples from the steroidogenesis assay were diluted with tris buffer (0.05 M tris HCl buffer with .01% bovine serum albumin). The sample/standard (100 µl) was then incubated with 100 µl of 40,000 dpm of 1,2,6,7-[^{3}H]-corticosterone (Radio-chemical Center, Amersham) and 200 µl of diluted anti-corticosterone serum at 4 °C for 16-24 hours.

At the end of incubation, 0.5 ml of an activiated charcoal suspension (0.25% Norit A and 0.25% Dextran T-70 in 0.05 M tris HCl buffer, pH 7.4) was added to each assay tube. After vortexing and standing on ice for 10 minutes, the tubes

were centrifuged at 4600 g for 10 minutes at 4 °C in a Beckman J2-21 centrifuge. An aliquot of 0.5 ml of the supernatant was mixed with 3.5 ml scintillant (12 gm PPO : 1.2 gm POPOP : 2 liters toluence : 1 liter triton X-100) and counted in a Beckman liquid scintillation counter (LS 1801) after vortexing. The rate of steroidogenesis is reported as the amount of corticosterone produced per hour per 25,000 adrenal decapsular cells.

3.1.3 Establishment of steroidogenesis assay system

3.1.3.1 Standard curve of corticosterone RIA

When rat decapsular adrenal cells were incubated with ACTH (section 3.1.2.2), corticosterone production in the cells was stimulated. To determine this stimulation of steroid production, the method used was radioimmunoassay for corticosterone (Li et al, 1982). Figure 3-1 presents a typical displacement curve of H-corticosterone using an anti-corticosterone serum purchased from Miles. As shown in figure 3-1, this assay could detect corticosterone within the range of 100 pg to 10 ng per tube and hence the amount of corticosterone produced by the decapsular adrenal cells could be accurately measured.

3.1.3.2 Selectivity of the steroidogenesis assay system

A response curve of rat adrenal decapsular cells to ACTH was shown in figure 3-2. A dose dependent response was observed in the dose range of 4×10^{-11} M to 4×10^{-9} M beyond

Figure 3-1

Standard curve of corticosterone RIA

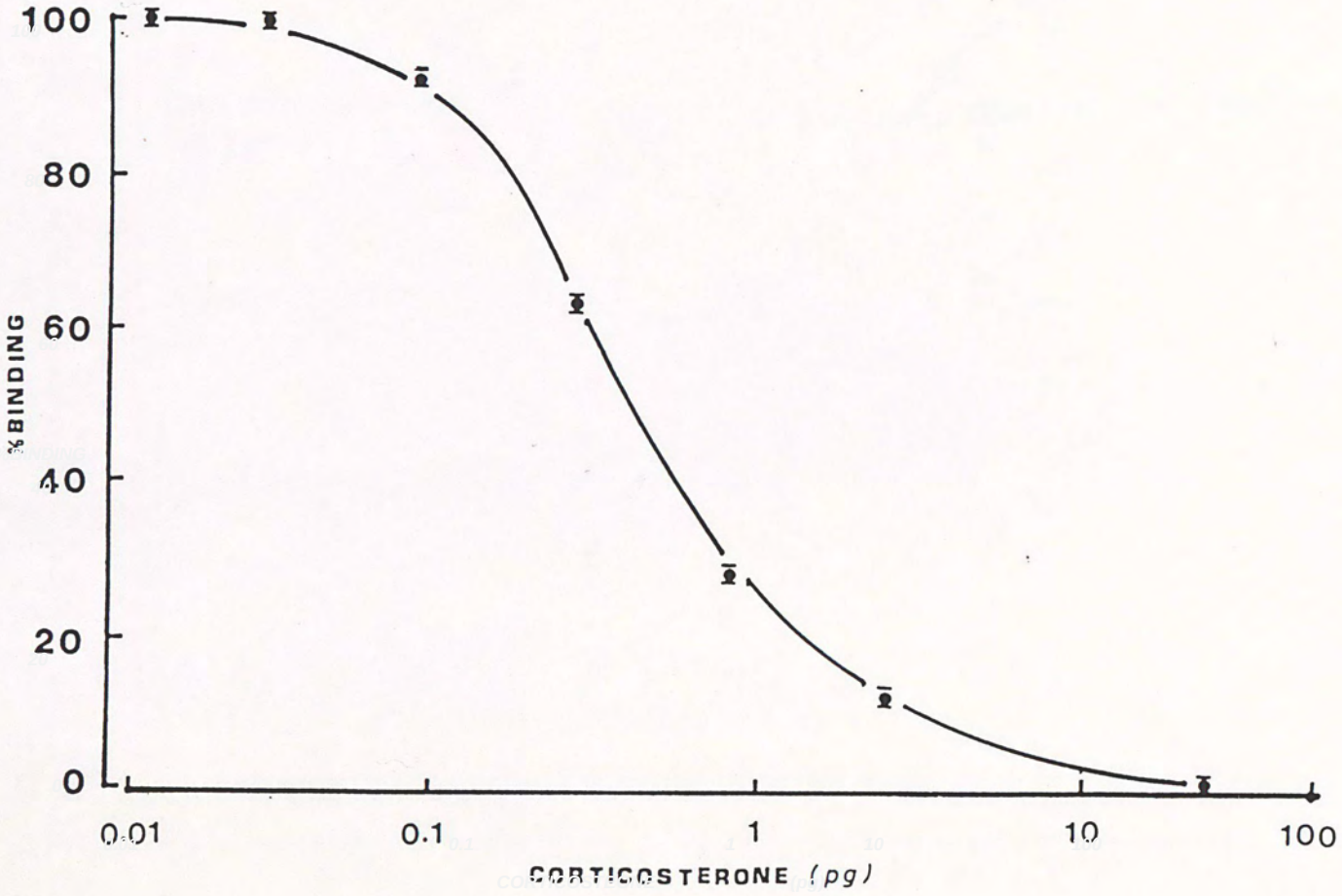
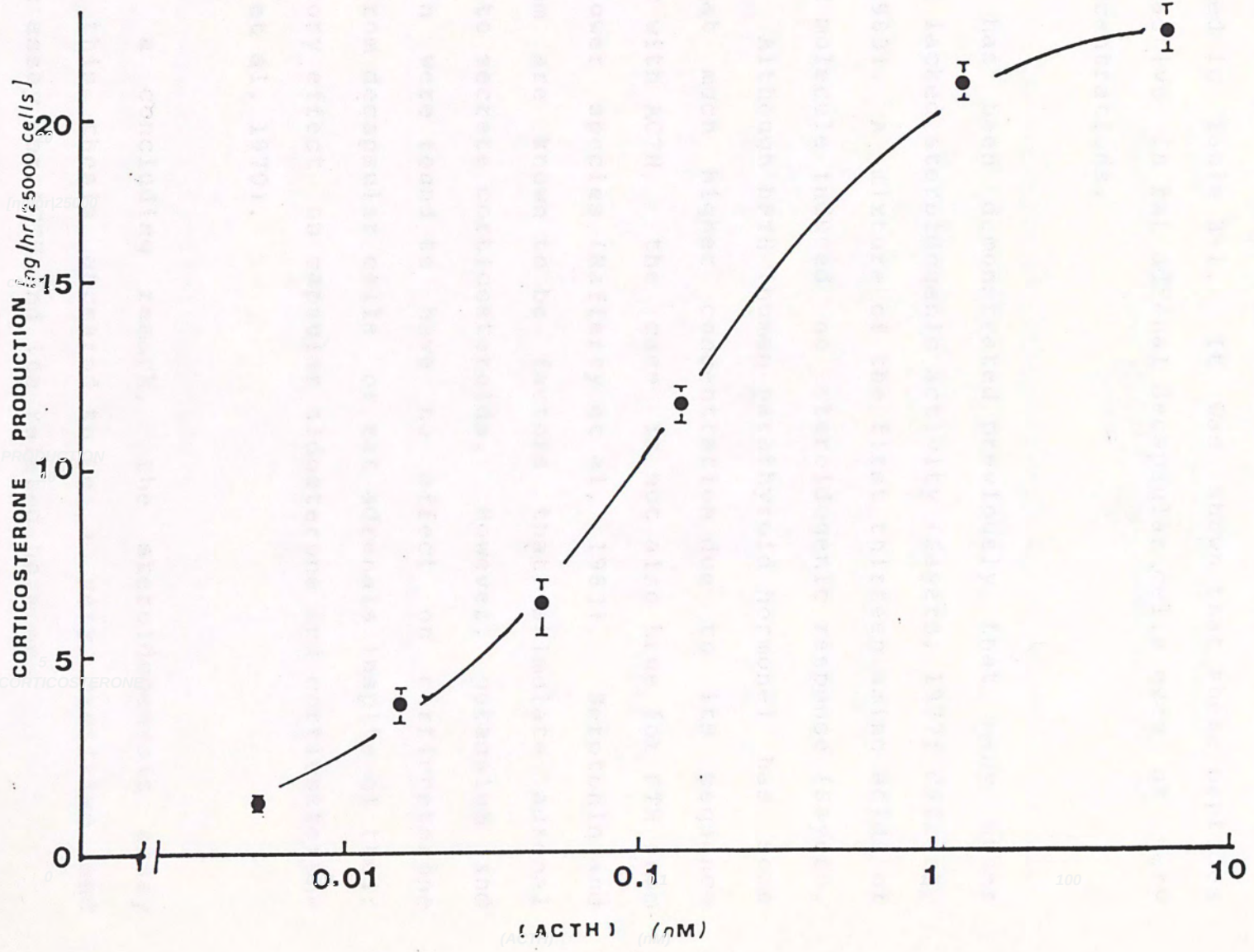

100
80
60
40
20
0
% BINDING
0.01
0.1
1
10
100
CORTICOSTERONE (pg)

Figure 3-2

Effect of porcine ACTH on steroidogenesis in rat adrenal decapsular cells


CORTICOSTERONE PRODUCTION (ng/hr/5000 cells)
0
5
10
15
20
0.01
0.1
1
10
(ACTH) (nM)

which the response leveled off. Besides ACTH and its analogs, other hormones known to have corticosteroidogenic activity were α-MSH and β-MSH but at dosages much higher than that of ACTH (Li et al, 1982). The effects of diverse peptides on steroidogenesis were investigated and the results were summarized in Table 3-1. It was shown that these peptides were inactive in rat adrenal decapsular cells even at very high concentrations.

It has been demonstrated previously that many other hormones lacked steroidogenic activity (Sayers, 1977; Rafferty et al, 1983). A mixture of the first thirteen amino acids of the ACTH molecule induced no steroidogenic response (Sayers, 1977). Although hPTH (human parathyroid hormone) has some effect at much higher concentration due to its sequence homology with ACTH, the case is not also true for PTH from other lower species (Rafferty et al, 1983). Serotonin and potassium are known to be factors that stimulate adrenal cortex to secrete corticosteroids. However, potassium and serotonin were found to have no effect on corticosterone output from decapsular cells of rat adrenals inspite of their stimulatory effect on capsular aldosterone and corticosterone (Hauing et al, 1970).

As a concluding remark, the steroidogenesis assay used in this thesis appeared to be a very sensitive and specific assay for ACTH and its related hormones.

Table 3-1. Steroidogenic activities of various peptides in rat adrenal decapsular cells

Hormone	Dose (μM)	Steroidogenic activity (ng corticosterone/hr/25,000 cells)
Control	0	UD
ACTH	4.0 x 10	4.38 ± 0.25 ^
β-Endorphin	1.0	UD
β-Lipotropin	0.2	UD
Dynorphin(1-13)	1.0	UD
Leu-enkephalin	10.0	1.74 ± 1.64
Met-enkephalin	10.0	UD
VIP	1.0	0.47 ± 0.09

Values represent mean ± S.E.M. of triplicate determinations.

VIP : Vasoactive intestinal peptide

UD : Undetectable

^ : $p < 0.001$ compared with control

3.2 Opiate receptor binding assay

3.2.1 Principle of opiate receptor binding assay

Immunohistochemical studies of endogenous opioid peptides have found that the three opioid peptide families are widely distributed in the rat brain, including the brain stem but not the cerebellum (Watson et al, 1984). In the rat brain homogenate, opiate receptor binding is enriched in the synaptosomal fraction which contains primarily pinched-off nerve-ending particles (Pert et al, 1974), although some activities are found also in the nuclear and microsomal fractions.

In the assay, D-ala^2-D-leu^5-(tyrosyl-3,5-[^{3}H])enkephalin, abbreviated as DADLE, was used to compete with the unknown samples for the specific opiate receptors on the rat brain synaptosomal membrane. The ability of the unknown to displace DADLE from the membrane was used as an index of its opiate activity. The extent of displacement was indicated by the amount of radioactivity of DADLE left on the membrane after incubation.

3.2.2 Method of opiate receptor binding assay

3.2.2.1 Preparation of the synaptosomal fraction from rat brains

The method of membrane preparation follows that of

Ferrara et al (1979) with slight modifications. Male Sprague-Dawley rats weighing 180-200 gm (about six weeks old) were decapitated and their brains rapidly removed and placed in chilled 0.32 M sucrose solution after removal of the cerebellums. The brains were then homogenized in 20 volumes of ice-cold 0.32 M sucrose solution by using a Polytron tissue disruptor (setting 4 for 20 seconds). The homogenate was centrifuged at 1000 g at 4 °C in a Beckman J2-21 centrifuge for 10 minutes to spin down the nuclear fraction. The supernatant was recentrifuged at 10,000 g at 4 °C for 30 minutes. The supernatant containing the microsomal fraction was discarded and the pellet washed twice with 20 volumes of fresh tris-HCl buffer. It is suggested that the synaptosomal fraction be used immediately or stored at -20 °C for not more than a few days.

3.2.2.2 Displacement of DADLE by unknown or standard from synaptosomal membrane

The prepared synaptosomal membrane was weighed and resuspended in 5 volumes (v/w) of 0.05 M tris HCl buffer (pH 7.4) at 4 C. The membrane (200 µl) was added to polystyrene tubes containing 100 µl opiate standard or samples, 100 ul protease inhibitor (bacitracin 0.5 mg/ml and trypsin inhibitor 0.5 mg/ml), and 100 µl DADLE, all in 0.05 M tris buffer, pH 7.4). After incubation at 4 °C for 16-24 hours, the reaction was stopped by filtration under low vacuum through glass fibre filters (Whatman GF/B). The tubes were washed twice and the filters once with 3 ml portions of ice-cold tris-HCl buffer. The filters were placed in scintillation vials and 5 ml of

scintillant (12 gm PPO and 1.2 gm POPOP in 2 litres toluene and 1 liter triton X-100) was added to each vial. After standing for at least half an hour, the radioactivity was measured by a Beckman liquid scintillation counter (LS 1801). The opiate receptor binding activity is computed as the percentage of DADLE bound to the brain membrane.

3.2.3 Establishment of opiate receptor binding assay system

3.2.3.1 Opiate receptors in rat brain

It is now believed that there are at least three and may be seven different types of opiate receptors (Yaksh and Noueihed, 1985; Kitchen, 1985). The distribution of endogenous opioid peptides in the rat brain has now been well-mapped out. Although all the three major families (POMC, pro-enkephalin and prodynorphin; section 1.3) are found in the rat brain, they exist as distinct opiate systems (Watson et al, 1984), which probably play different physiological roles. However, only the μ-receptor has been extracted from the rat brain (Cho et al, 1986).

3.2.3.2 Selectivity of the opiate receptor assay system

Each endogenous opioid peptide has its own selectivity to different types of opiate receptors (Hughes, 1984), e.g. β-endorphin prefers μ- and δ-receptors, met- and leu-enkephalins prefer δ- to μ-receptors but dynorphins prefer k-receptor but not the other two. The radioactive molecule, DADLE, a leu-enkephalin analogue, was used in the opiate receptor binding

as the ligand. As its natural analogue, DADLE prefers to bind δ-receptors so our assay system is in fact established to test the presence of opioid peptides with δ-preference in tissue extracts and chromatographic fractions.

Other opioid peptides including β-endorphin, dynorphin (1-13) and met-enkephalin, were also tested by the same method. The resulting competition curves could all be well fitted into the single site model as shown in figure 3-3 and the Kd values of them were found to be quite different from one another as listed in table 3-2. As predicted, the opioid of the enkephalin familis, leu- and met-enkephalins, were found to be more potent than β-endorphin and dynorphin in the displacement of DADLE from its binding sites indicating different receptor systems for them.

Figure 3-3 shows a displacement curve of DADLE by leu-enkephalin. It was a mono-component saturable binding curve with an apparent Kd of 29.0 nM indicating that DADLE was displaced apparently from a single type of binding sites. Since peptide bonds involving amino acids of the D-configuration are very unlikely to be broken by common peptidases, the displacement of DADLE by tissue extracts or fractions are unlikely to be due to the degradation of this labelled peptide by enzymes present in the trissue extracts or fractions. However, protease inhibitors such as bracitracin and trypsin inhibitor were still added to deactivate any possible protease activities.

Figure 3-3

Inhibition of [^{3}H] DADLE binding to rat brain membranes by met-enkephalin (1), leu-enkephalin (2), β-endorphin (3) and dynorphin (4)

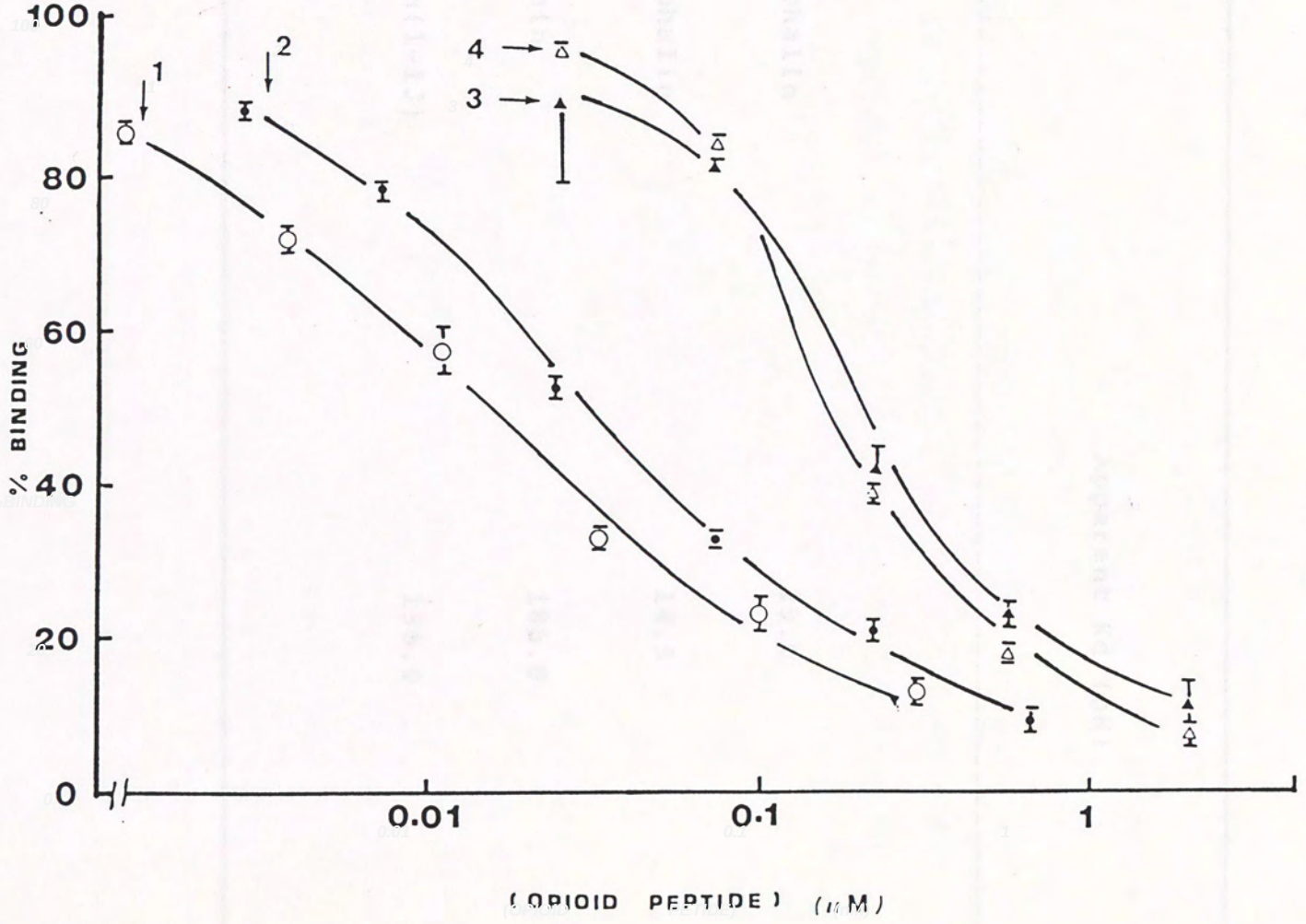

100
80
60
40
20
0
% BINDING
1
2
3
4
0.01
0.1
1
(OPIOID PEPTIDE) (μM)

Table 3-2. Apparent Kd values of opiates for DADLE binding site in rat brain membrane

Opiate	Apparent Kd (nM)
Leu-enkephalin	29.0
Met-enkephalin	14.5
β-Endorphin	185.0
Dynorphin(1-13)	195.0

3.3 ß-Endorphin radioimmunoassay (RIA)

The high specificity of antibody can be used to distinguish among individual opioid peptides which are indistinguishable in the opiate receptor binding assay. ß-Endorphin but not any other opioid peptide was chosen as the hormone to be studied because it shares a common precursor, POMC, with ACTH in higher vertebrates. The presence of both ß-endorphin and ACTH in the same tissue will give an insight into the presence of a possible local syntheszing system.

3.3.1 Principle of ß-endorphin RIA

The assay makes use of the competitive binding between the (3-[^{125}I]iodotyrosyl^{27})ß-endorphin (human) and the test material to the ß-endorphin antiserum (raised in rabbit). The amount of ß-endorphin immunoreactivity was calculated from a standard curve constructed using human ß-endorphin. The radioactive ß-endorphin used has been proved to bind to rat brain synaptosomal membrane and displaced by unlabelled peptide.

3.3.2 Method of ß-endorphin RIA

The assay buffer used in the ß-endorphin RIA was a phosphate buffer consisting of 50 mM sodium phosphate, 0.2% bovine serum albumin (BSA), 10 mM EDTA, 2 mg/100 ml trypsin

inhibitor and 0.01% thimerosal. The resulting solution was adjusted to pH 7.4. The volume of the test sample was made up to 600 µl in the assay buffer. The standard curve was constructed with a serial dilution of human β-endorphin in 600 µl. Then 100 µl of antiserum (Amersham's code N.1621) of β-endorphin was added into each tube except the blank to which 100 µl of assay buffer was added. The tubes were then incubated for 24 hrs at 4 °C before the 100 µl of 3-[^{125}I]iodotyrosyl^{27})β-endorphin (30,000 dpm/ml, Amersham) was added. After that, the solution was incubated for another 24 hrs at 4 °C. After the incubation, a suspension of 500 µl activated charcoad solution (1.0 gm Norit A and 0.1 gm dextran T-70 in 50 ml assay buffer; stirred for at least 30 minutes at 4 °C just before use) was added to each tube which were then centrifuged at 4000 g for 10 minutes after vortexing and standing for five minutes at 4 °C. An aliquot of 10 ml of supernatant (containing the bound hormone) was taken and counted in a γ-counter for 120 seconds. The results are calculated as the percentage of [^{125}I]β-endorphin bound to the antiserum.

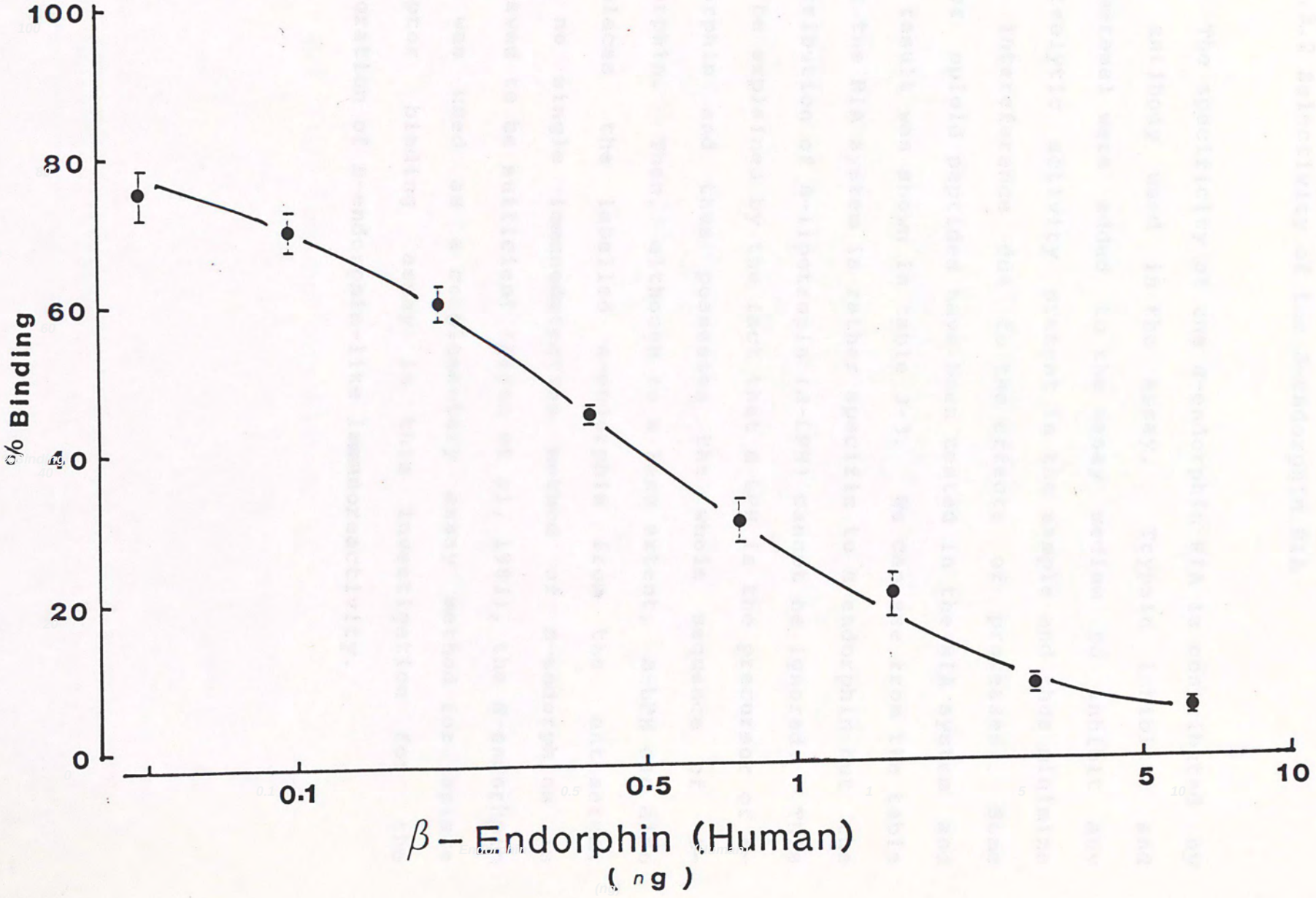
3.3.3 Establishment of the β-endorphin RIA system

3.3.3.1 Standard curve of β-endorphin RIA

The standard curve of β-endorphin RIA using standard human β-endorphin is shown in figure 3-4. It looks much like the one in corticosterone RIA (figure 3-1). The percentage binding of sample was calculated by the same method and the amount of β-endorphin immunoreactivity in the sample was

Figure 3-4

Standard curve of β-endorphin RIA


100
80
60
40
20
0
% Binding
0.1
0.5
1
5
10
β – Endorphin (Human)
(ng)

obtained directly from the standard curve of β-endorphin.

3.3.3.2 Selectivity of the β-endorphin RIA

The specificity of the β-endorphin RIA is contributed by the antibody used in the assay. Trypsin inhibitor and thimerosal were added to the assay medium to inhibit any proteolytic activity present in the sample and thus minimize any interference due to the effects of proteases. Some other opioid peptides have been tested in the RIA system and the result was shown in table 3-3. We can see from the table that the RIA system is rather specific to β-endorphin but the contribution of β-lipotropin (β-LPH) cannot be ignored. This can be explained by the fact that β-LPH is the precursor of β-endorphin and thus possesses the whole sequence of β-endorphin. Then, although to a less extent, β-LPH can also displaces the labelled β-endorphin from the antiserum. As no single immunodetection method of β-endorphins is believed to be sufficient (Bayon et al, 1983), the β-endorhpin RIA was used as a complementary assay method for opiate receptor binding assay in this investigation for the exploration of β-endorphin-like immunoreactivity.

Table 3-3. ß-Endorphin immunoreactivity of various peptides in the ß-endorphin RIA

Hormone	Dose (ng)	ß-Endorphin eq (ng)
ß-Endorphin	1	1
ß-Lipotropin	20	5.06
Dynorphin(1-13)	56	UD
Leu-enkephalin	25000	UD
Met-enkephalin	25000	UD

Values represent mean ± S.E.M. of triplicate determinations.

UD : Undetectable

^ : $p < 0.001$ compared with control

DATA AND RESULT FROM CLASS MAMMALIA

- Hearts, pancreas and testis

  from various mammals

Chapter 4 DATA AND RESULT FROM CLASS MAMMALIA - Hearts, pancreas and testes from various mammalian species

As discussed in Chapter 1, opiates and ACTH have been found in many different mammalian tissues (section 1.2 & 1.3) besides pituitary and brain, ACTH and endogenous opioid peptides are widely distributed in different organs of various mammalian species. In this thesis, the tissues of some mammals were studied to investigate the presence of ACTH- and opiate-like activities.

4.1 Mammalian hearts

4.1.1 Introduction - ACTH and opiates in rodent hearts

Studies had been conducted by different groups to search for ACTH and endogenous opiates in the hearts of Rodentia. The existence of immunoreactive ACTH (Saito et al, 1983) and dynorphin (Spampinato and Goldstein, 1983) in the rat heart, and met- and leu-enkephalin (Lang et al, 1983) in the guinea pig heart have been reported. In this chapter, two other mammals in Rodentia - the gerbil and the hamster, were studied together with the rat and the guinea pig to see whether the case is the same for all of them. The opiate receptor binding assay and the β-endorphin RIA were used to monitor opiate-like activity in the chromatographic fractions.

4.1.2 Materials and methods - extraction of rodent hearts

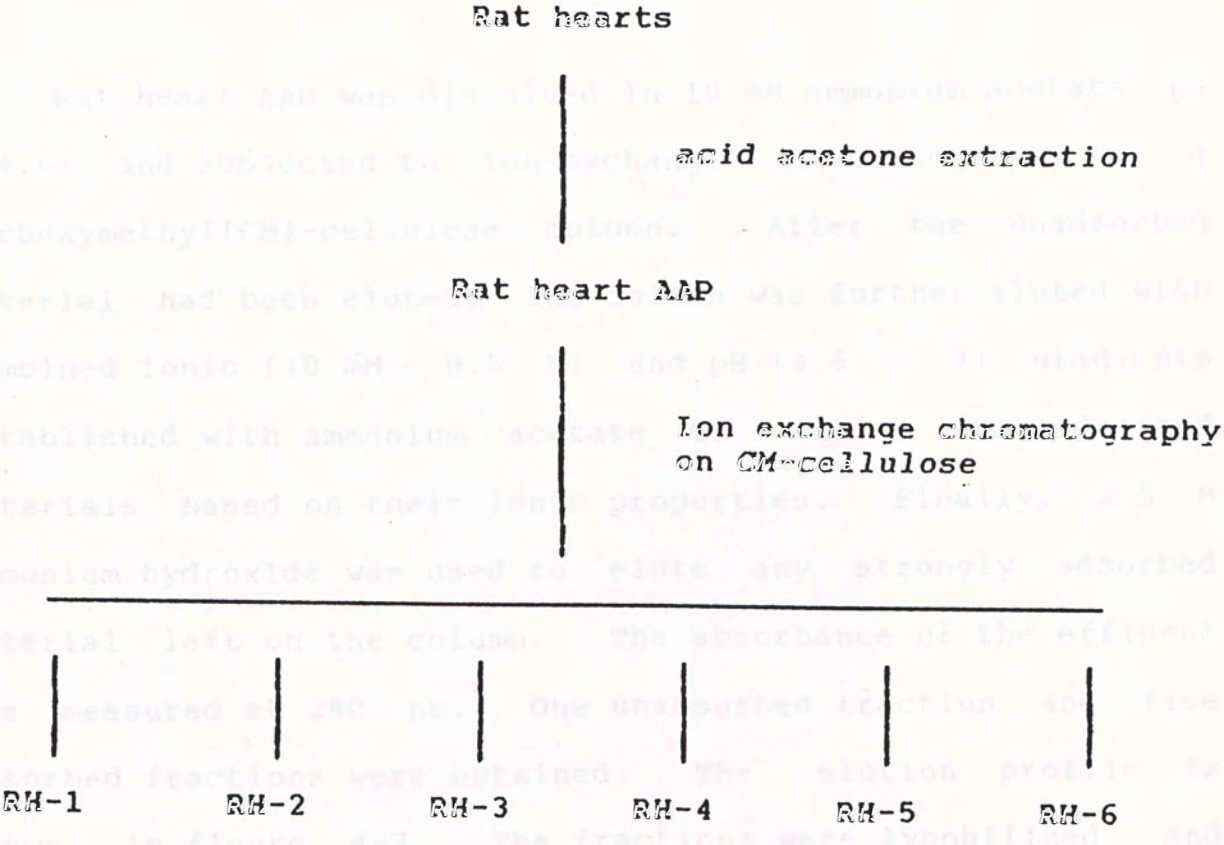
4.1.2.1 Extraction of rat hearts

The protocol for the extraction of rat hearts is shown in figure 4-1. Rat heart acid acetone powder (AAP) was first prepared from rat hearts and then the AAP was loaded on a carboxymethyl (CM)-cellulose column.

4.1.2.1a Preparation of acid acetone powder (AAP) of rat heart

The hearts were collected from adult rats after scrificed by cervical dislocation, and stored forzen at - 70 °C until use. First, the hearts were rinsed in 0.9% saline at 4 °C to remove blood. The tissues were then cut into pieces and extracted with 2 vol. (w/v) of a mixture of acetone-water-HCl (600 : 75 : 15) in a blender at 4 °C. The resulting mixture was stirred overnight. After centrifugation at 10,000 g for 30 min. at 4 °C, the supernatant was saved and the pellet was re-extracted with 1 vol. of 80% acetone. The extract was then stirred for 30 min. After centrifugation at 10,000 g for another 30 min. at 4 °C, the supernatants from the two centrifugation steps were pooled and added with stirring to 5 vol. (v/v) of acetone which had been chilled at 4 °C for at least 24 hr. The final mixture was again allowed to stand overnight. The precipitate was collected on a circle of Whatman No. 1 filter paper, washed twice with prechilled acetone and dried under vacuum. After lyophilization, the product, known as acid acetone powder (AAP), was kept at -20 °C

Figure 4-1 Extraction and purification of ACTH-like and opiate-like activities from of rat hearts



until further processing.

4.1.2.1b Purification of rat heart AAP by ion-exchange chromagraphy

Rat heart AAP was dissolved in 10 mM ammonium acetate (pH = 4.6) and subjected to ion-exchange chromatography on a carboxymethyl(CM)-cellulose column. After the unadsorbed material had been eluted, the column was further eluted with combined ionic (10 mM - 0.5 M) and pH (4.6 - 7) gradients established with ammonium acetate to recover the adsorbed materials based on their ionic properties. Finally, 2.5 M ammonium hydroxide was used to elute any strongly adsorbed material left on the column. The absorbance of the effluent was measured at 280 nm. One unadsorbed fraction and five adsorbed fractions were obtained. The elution profile is shown in figure 4-2. The fractions were lyophilized and stored at -20 °C until assay for steroidogenic and opiate receptor binding activities.

4.1.2.2 Extraction of hamster and guinea pig hearts

The protocols for the extraction of hearts of the two mammalian species are shown in figure 4-3, 4-4. The acid acetone powders (AAPs) of the hearts were first preparated from the hearts obtained from the two species and then the AAPs were loaded on a Sephadex G-25 column.

4.1.2.2a Preparation of AAP of hamster and guinea pig hearts

Figure 4-2

Ion exchange chromatography of rat heart AAP on a CM-cellulose column (1.4 x 36 cm). The AAP (500 gm) was dissolved in starting buffer, centrifuged and the resulting supernatant applied on column. Fraction size = 5 ml.

Buffer: 10 mM NH_4OAc, pH 4.6 (fractions 1-20);
10 - 100 mM NH_4OAc, pH 4.6 - 6.7 (fractions 21-60);
0.1 - 0.2 M NH_4OAc, pH 6.7 - 7 (fractions 61-100);
0.2 - 0.5 M NH_4OAc, pH 7 (fractions 101-140);
0.5 M NH_4OAc, pH 7 (fractions 141-160);
12.5 M NH_4OH, pH 11 (fractins 161-183).

Yields: RH-1, 41 mg;
RH-2, 11.6 mg;
RH-3, 25.3 mg;
RH-4, 7.2 mg;
RH-5, 17.8 mg;
RH-6, 56.6 mg.

(1) : pH gradient
(2) : ionic gradient
(M) : Molarity of eluent

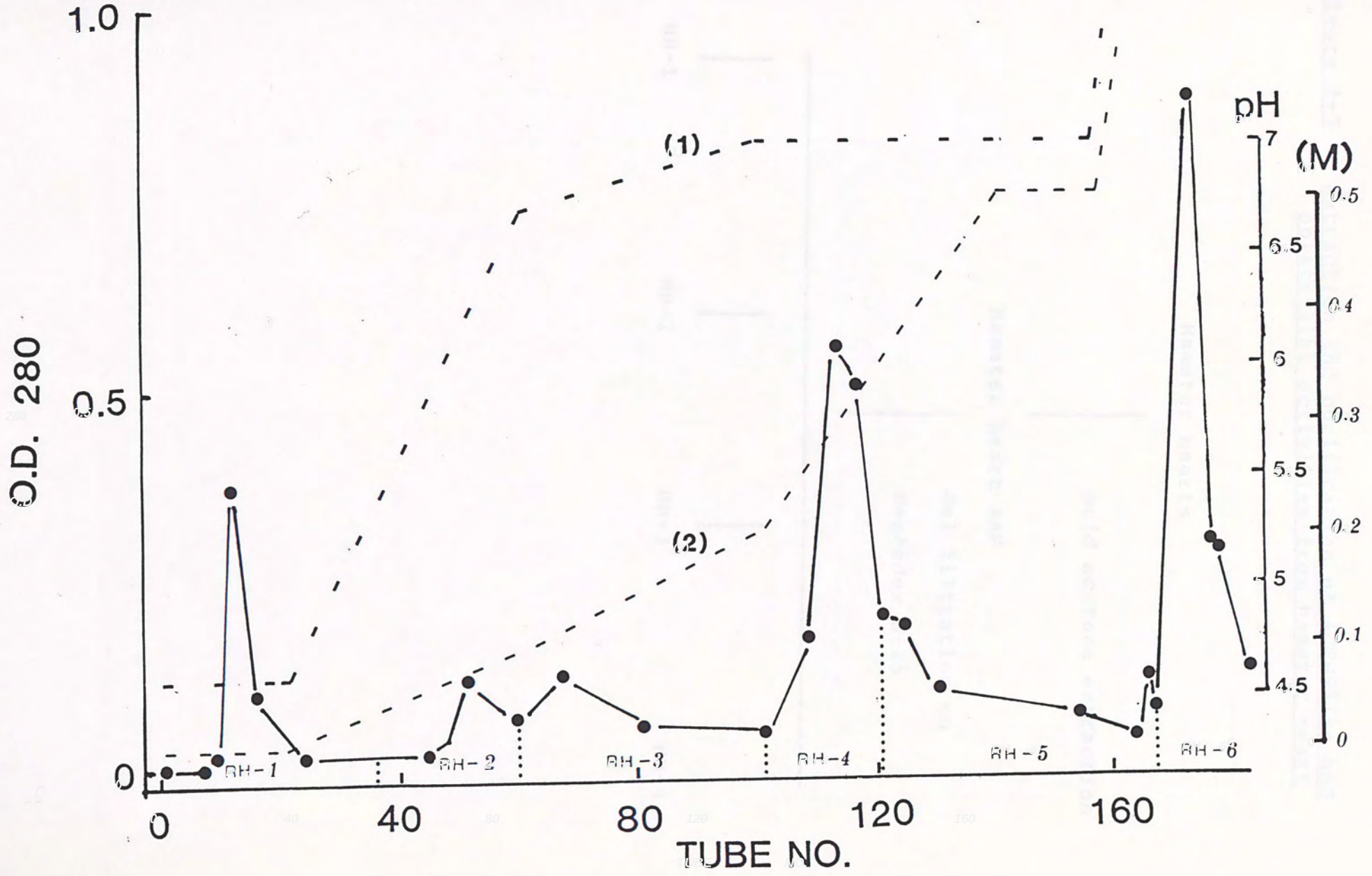

1.0
0.5
0
O.D. 280
(1)
(2)
pH
7
6.5
6
5.5
5
4.5
(M)
0.5
0.4
0.3
0.2
0.1
0
RH-1
RH-2
RH-3
RH-4
RH-5
RH-6
0
40
80
120
160
TUBE NO.

Figure 4-3 Extraction and purification of ACTH-like and opiate-like activities from hamster heart

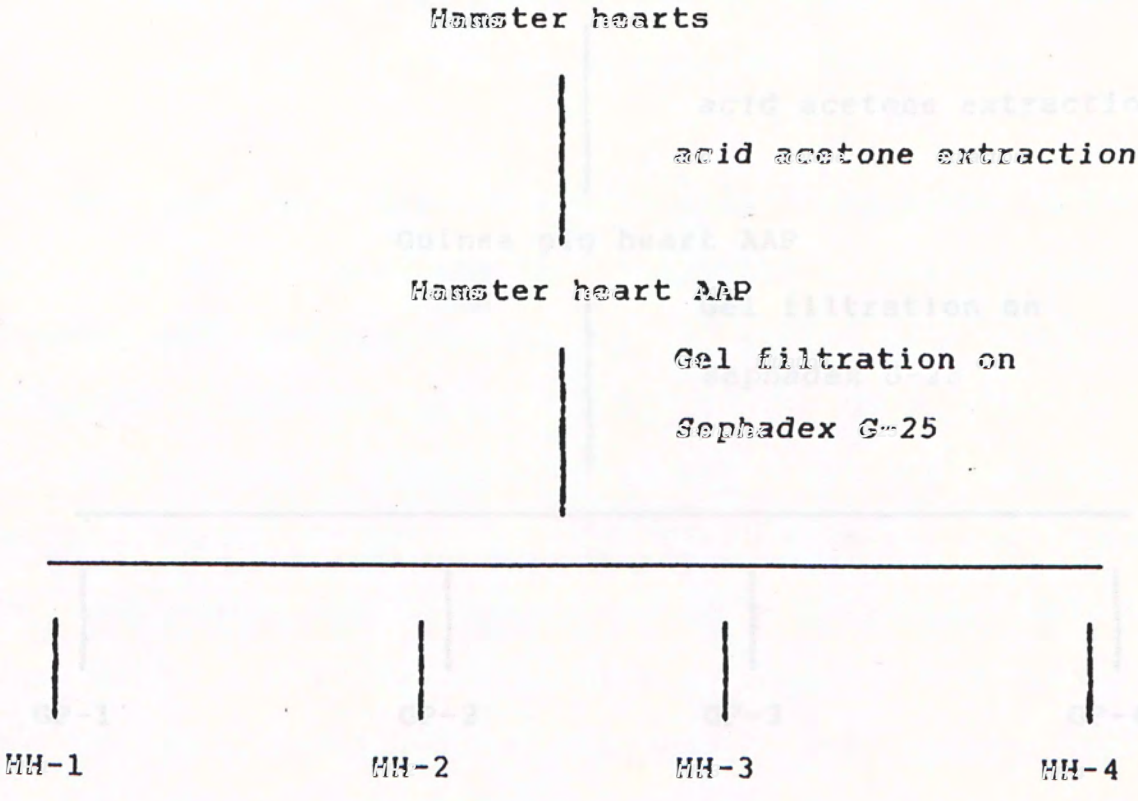
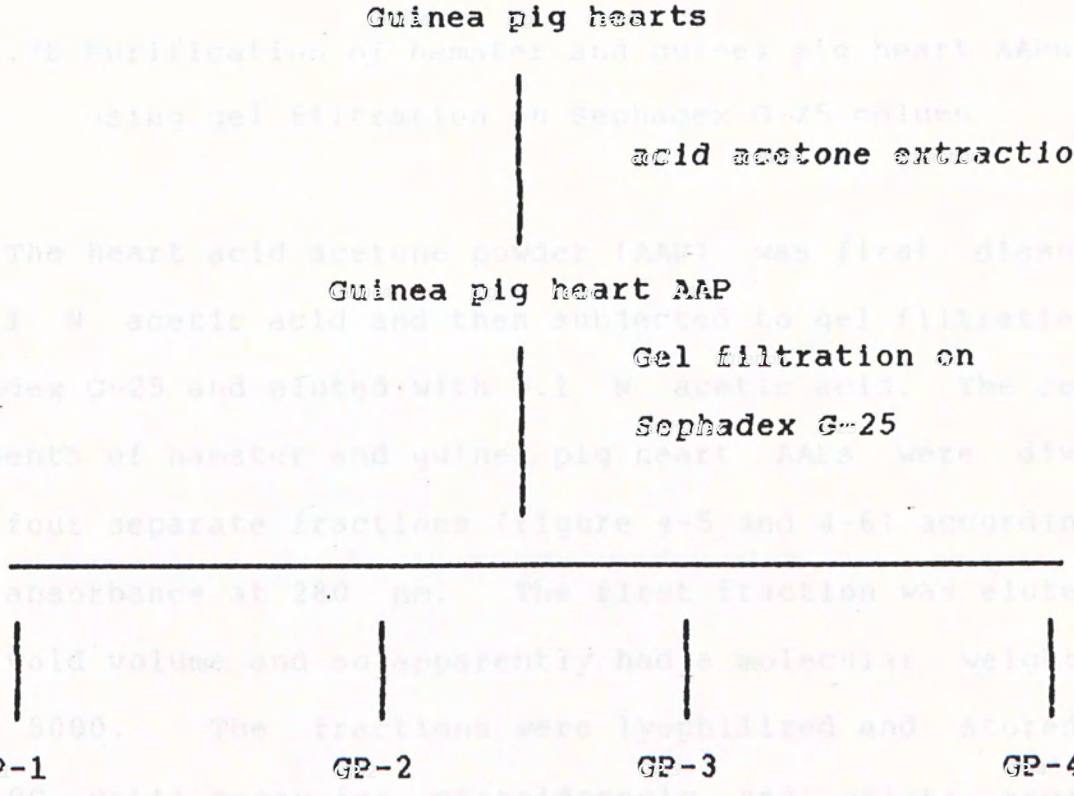


Figure 4-4 Extraction and purification of ACTH-like and opiate-like activities from guinea pig hearts



The AAP of hamster and guinea pig hearts were prepared with the method used for the preparation of rat heart AAP (section 4.1.2.1a).

4.1.2.2b Purification of hamster and guinea pig heart AAPs using gel filtration on Sephadex G-25 column

The heart acid acetone powder (AAP) was first dissolved in 0.1 N acetic acid and then subjected to gel filtration on Sephadex G-25 and eluted with 0.1 N acetic acid. The column effluents of hamster and guinea pig heart AAPs were divided into four separate fractions (figure 4-5 and 4-6) according to the absorbance at 280 nm. The first fraction was eluted at the void volume and so apparently had a molecular weight of over 5000. The fractions were lyophilized and stored at -20 °C until assay for steroidogenic and opiate receptor binding activities.

4.1.2.3 Extraction of gerbil hearts

The protocol for the extraction of gerbil heart is shown in figure 4-7. The acid acetone powder (AAP) of the hearts was first prepared from gerbil heart. After salt fractionation, the extract was desalted on a Sephadex G-10 column. After that, a CM-cellulose column was used for further purification of the resulting fractions.

4.1.2.3a Preparation of AAP of gerbil hearts

The AAP of gerbil heart was prepared with the method used

Figure 4-5

Gel filtration of hamster heart AAP on a Sephadex G-25 column (3.5 x 80 cm). The AAP (500 mg) was dissolved in buffer, centrifuged and the resulting supernatant applied on column. Fraction size = 4 ml.

Buffer: 0.1 M acetic acid.

Yields: HH-1, 177.3 mg; (Void volume)
HH-2, 56.9 mg;
HH-3, 25 mg;
HH-4, trace amount.

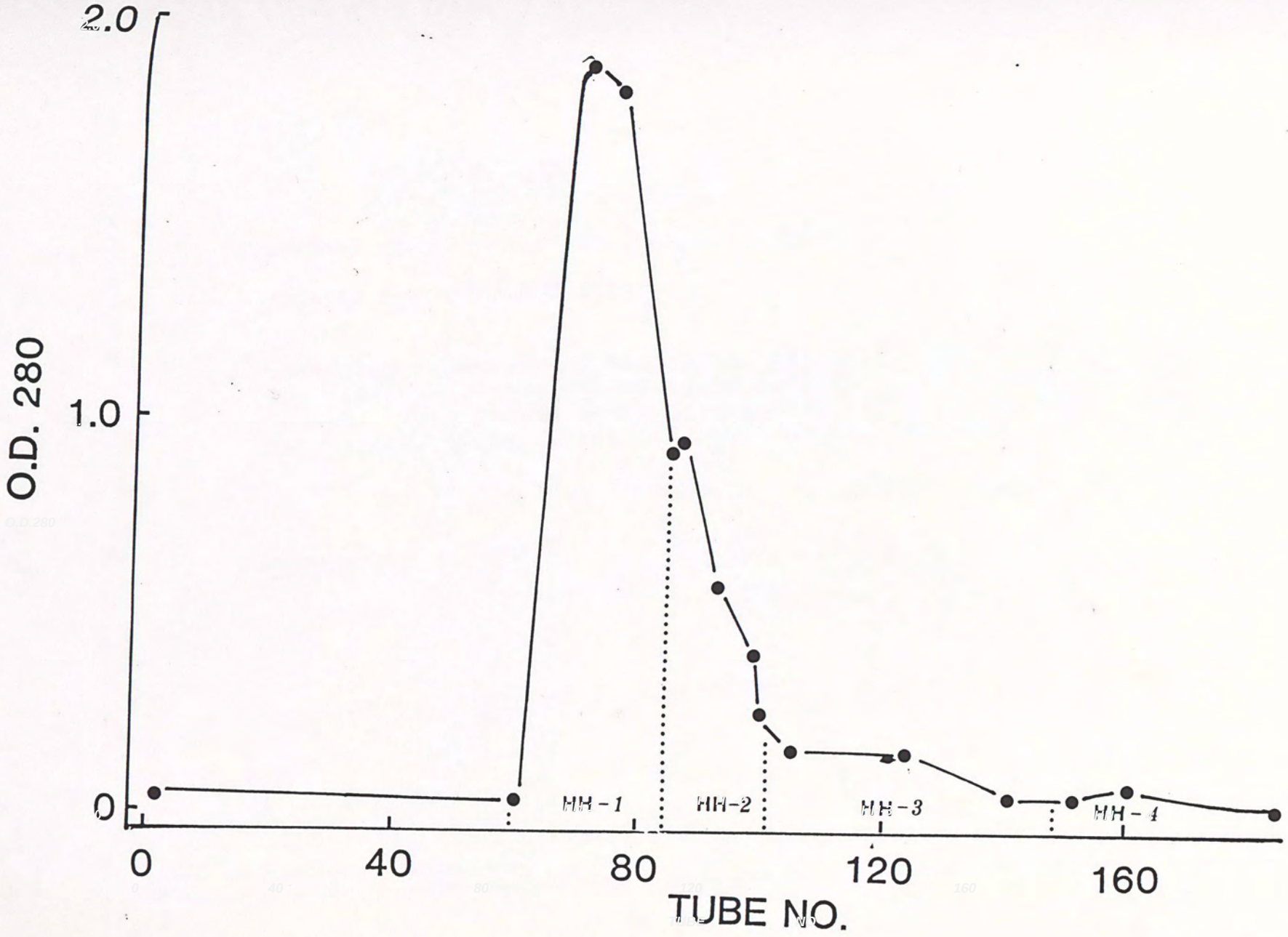

2.0
1.0
0
O.D. 280
HH-1
HH-2
HH-3
HH-4
0
40
80
120
160
TUBE NO.

Figure 4-6

Gel filtration of guinea pig heart AAP on Sephadex G-25 (3.5 x 80 cm). AAP on a Sephadex G-25 column (3.5 x 80 cm). The AAP (500 mg) was dissolved in buffer, centrifuged and the resulting supernatant applied on column. Fraction size = 5 ml.

Buffer: 0.1 M acetic acid.

Yields: GP-1, 141.3 mg; (Void volume)
GP-2, 80.4 mg;
GP-3, 205 mg;
GP-4, 31.6 mg.

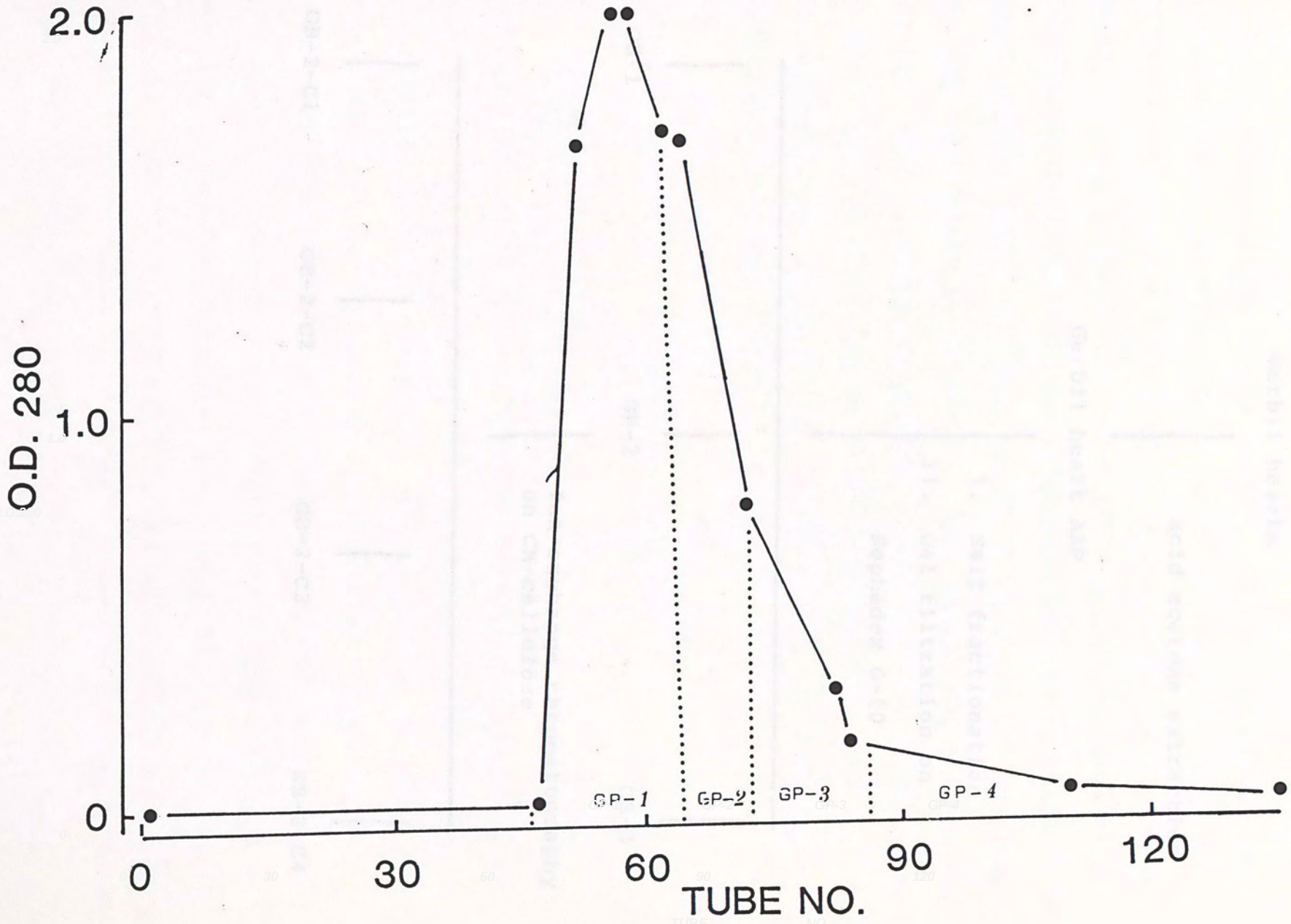
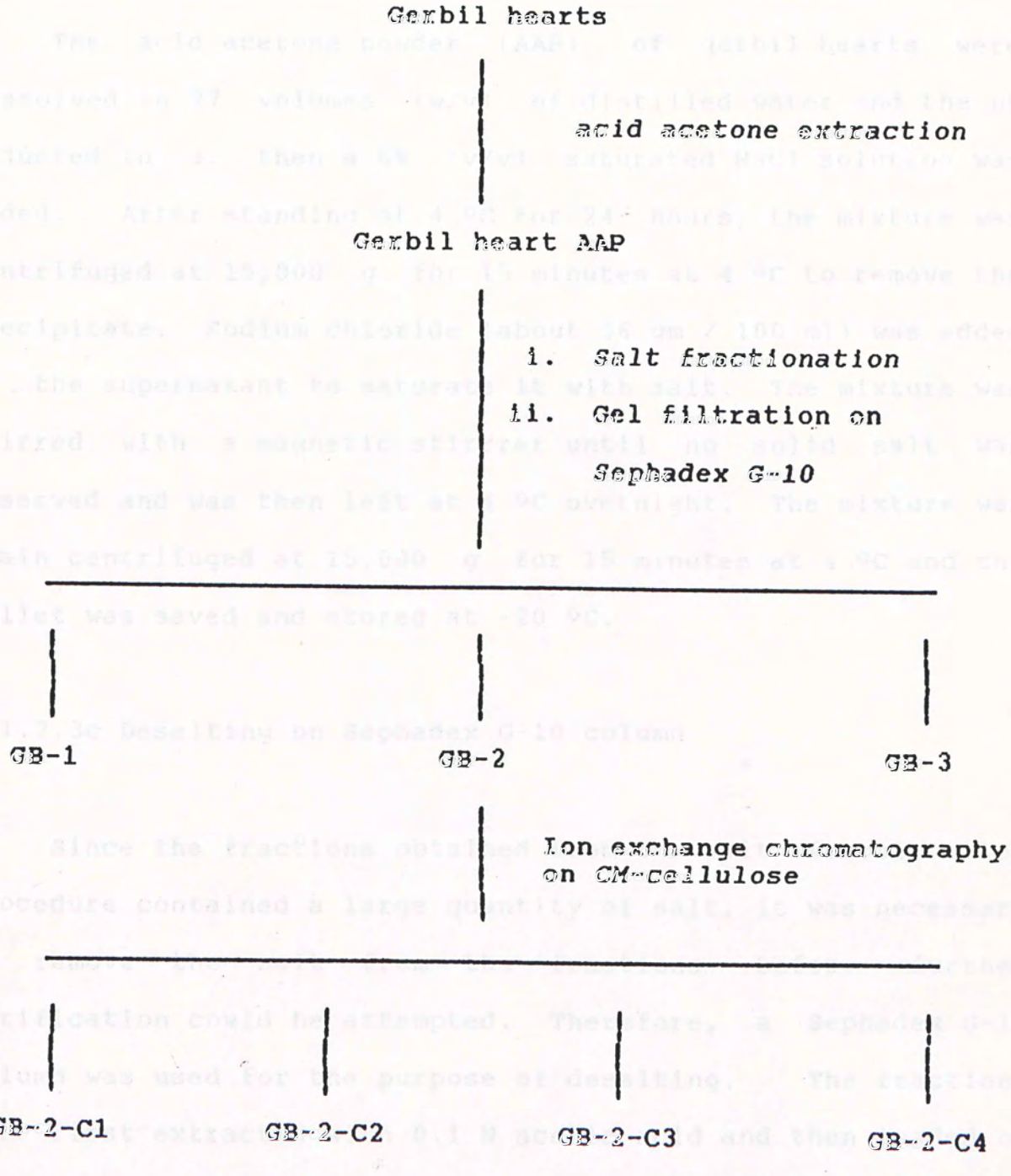

2.0
1.0
0
O.D. 280
GP-1
GP-2
GP-3
GP-4
0
30
60
90
120
TUBE NO.

Figure 4-7 Extraction and purification of ACTH-like and opiate-like activities from gerbil heart



for the preparation of rat heart AAP (section 4.1.2.1a).

4.1.2.3b Salt fractionation of gerbil heart AAP

The acid acetone powder (AAP) of gerbil hearts were dissolved in 27 volumes (w/v) of distilled water and the pH adjusted to 3. then a 6% (v/v) saturated NaCl solution was added. After standing at 4 °C for 24 hours, the mixture was centrifuged at 15,000 g for 15 minutes at 4 °C to remove the precipitate. Sodium chloride (about 36 gm / 100 ml) was added to the supernatant to saturate it with salt. The mixture was stirred with a magnetic stirrer until no solid salt was observed and was then left at 4 °C overnight. The mixture was again centrifuged at 15,000 g for 15 minutes at 4 °C and the pellet was saved and stored at -20 °C.

4.1.2.3c Desalting on Sephadex G-10 column

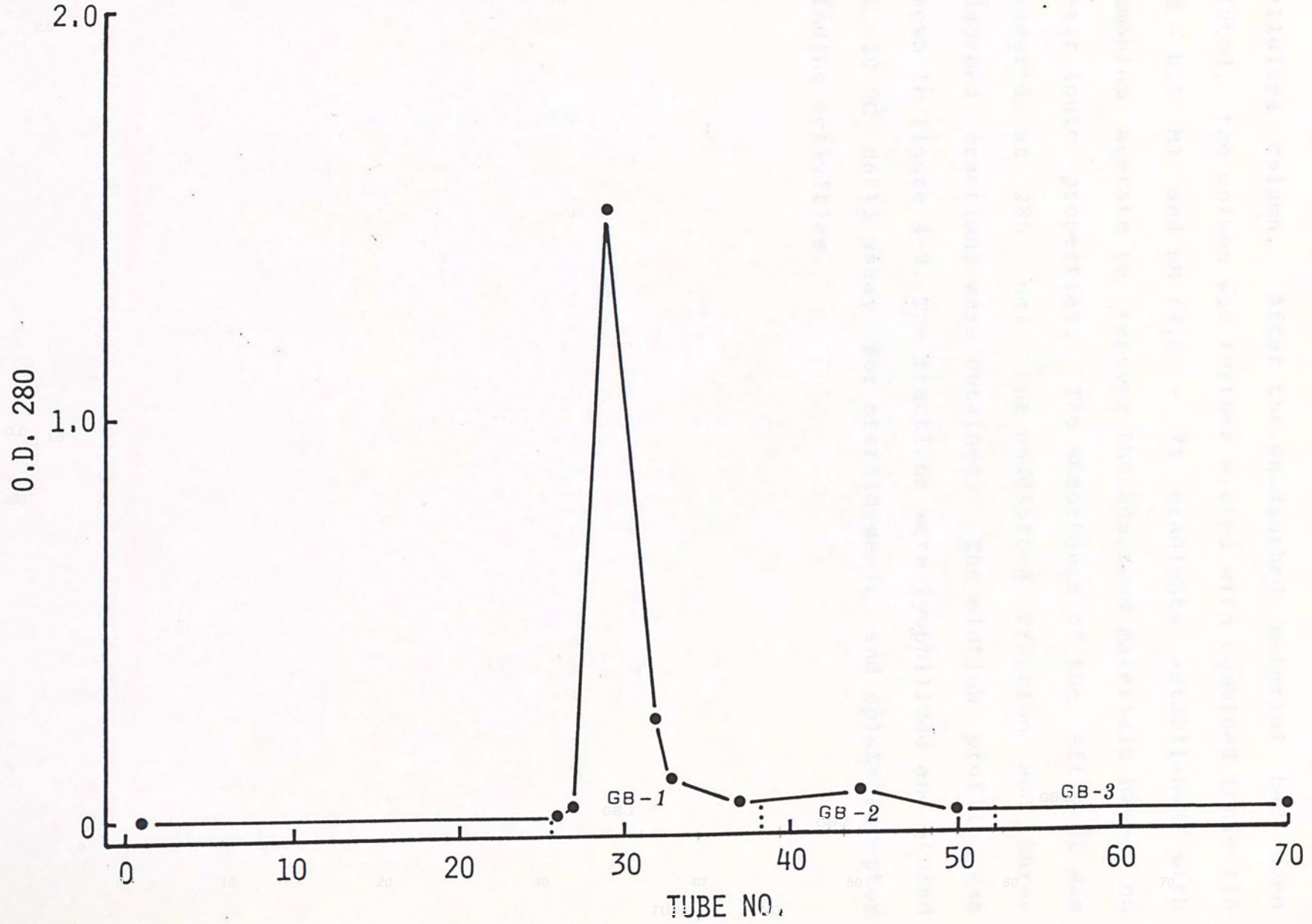
Since the fractions obtained from the salt fractionation procedure contained a large quantity of salt, it was necessary to remove the salt from the fractions before further purification could be attempted. Therefore, a Sephadex G-10 column was used for the purpose of desalting. The fractions were first extracted with 0.1 N acetic acid and then loaded on the G-10 column. Three fractions were eluted (figure 4-8). The first desalted fraction was found to contain opiate activity but no steroidogenic activity although both activities were found in the gerbil heart AAP. The second fraction was loaded on a CM-cellulose column.

Figure 4-8

Guinea pig heart AAP (1.18 g) subjected to salt fractionation and then desalted on a Sephadex G-10 (3 x 80 cm) column. Fraction size = 5 ml.

Buffer: 0.1 M acetic acid.

Yields: GB-1, 35 mg; (Void volume)
GB-2, 276.8 mg;
GB-3, trace amount.


2.0
1.0
0
O.D. 280
0
10
20
30
40
50
60
70
TUBE NO.
GB-1
GB-2
GB-3

4.1.2.3d Purification by ion-exchange chromagraphy

The fraction was dissolved in 10 mM ammonium acetate (pH = 4.6) and subjected to ion-exchange chromatography on a CM-cellulose column. After the unadsorbed material had been eluted, the column was further eluted with combined ionic (10 mM - 0.5 M) and pH (4.6 - 7) gradients established with ammonium acetate to recover the adsorbed materials based on their ionic properties. The absorbance of the effluent was measured at 280 nm. One unadsorbed fraction and three adsorbed fractions were obtained. The elution profile was shown in figure 4-9. The fractions were lyophilized and stored at -20 °C until assay for steroidogenic and opiate receptor binding activities.

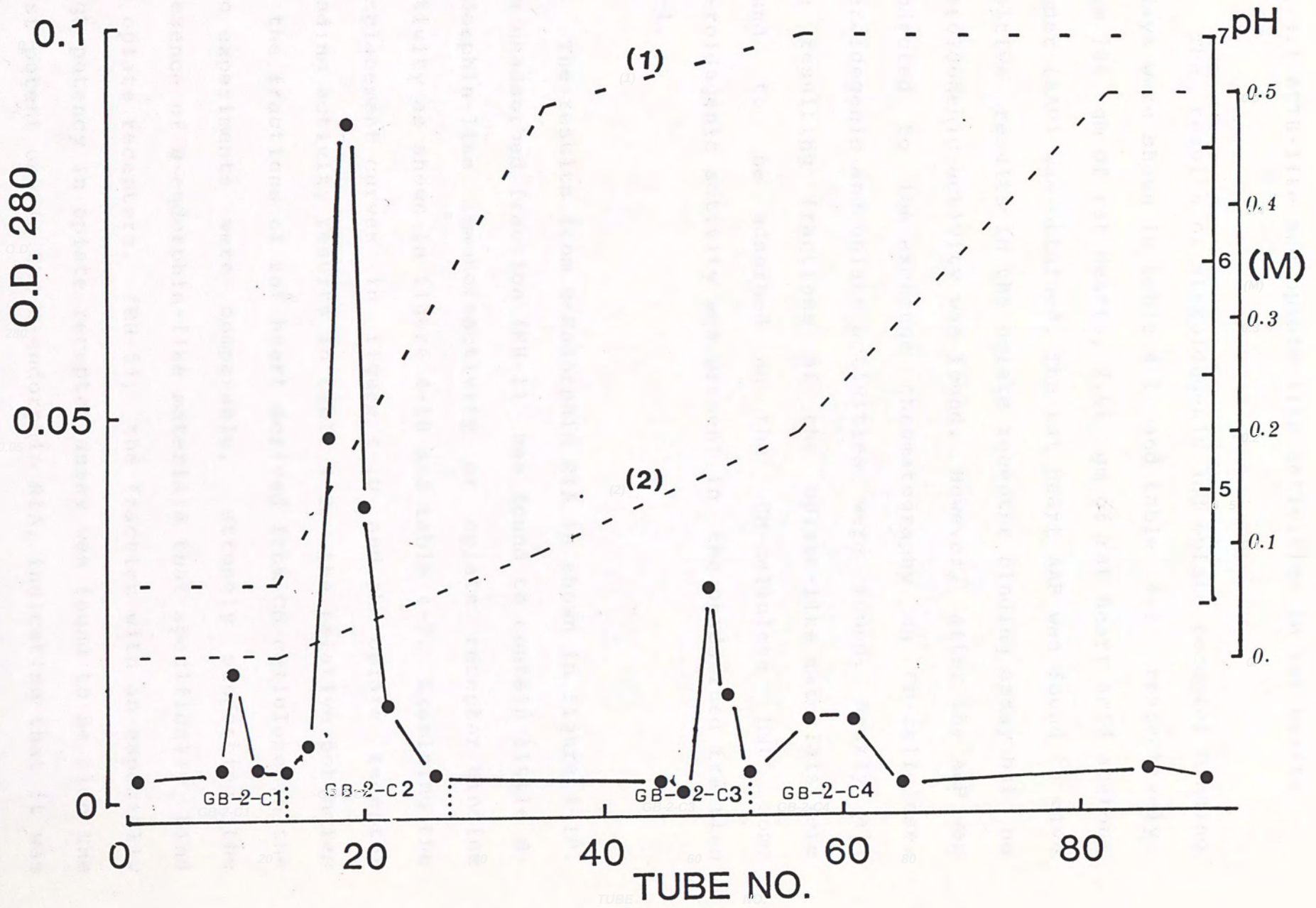
Figure 4-9

Ion exchange chromatography of GB-2 (277 mg) on CM-cellulose (1.4 x 36 cm). Fraction size = 5 ml.

Buffer: 10 mM NH_4OAc, pH 4.6 (fractions 1-12);
10 - 100 mM NH_4OAc, pH 4.6 - 6.7 (fractions 13-35);
0.1 - 0.2 M, NH_4OAc, pH 6.7 - 7 (fractions 36-56);
0.2 - 0.5 M, NH_4OAc, pH 7 (fractions 57-82).
0.5 M NH_4OAc, pH 7 (fractions 83-93)

Yields: GB-2-C1, 1.6 mg; (Void volume)
GB-2-C2, 183.4 mg;
GB-2-C3, 55 mg;
GB-2-C4, trace amount.

(1) : pH gradient
(2) : ionic gradient
(M) : Molarity of eluent


0.1
0.05
0
O.D. 280
(1)
(2)
pH
7
6
5
(M)
0.5
0.4
0.3
0.2
0.1
0.
GB-2-C1
GB-2-C2
GB-2-C3
GB-2-C4
0
20
40
60
80
TUBE NO.

4.1.3 Results : rodent hearts

4.1.3.1 ACTH-like and opiate-like activities in rat hearts

The results of steroidogenic and opiate receptor binding assays were shown in table 4-1 and table 4-2 respectively. From 166 gm of rat hearts, 2.66 gm of rat heart acid acetone powder (AAP) was obtained. The rat heart AAP was found to give positive results in the opiate receptor binding assay but no steroidogenic activity was found. However, after the AAP was subjected to ion-exchange chromatography on CM-cellulose, steroidogenic and opiate activities were found. Nearly all the resulting fractions of the opiate-like materials were found to be adsorbed on the CM-cellulose but some steroidogenic activity was present in the unadsorbed fraction RH-1.

The results from ß-Endorphin RIA is shown in figure 4-10. The unadsorbed fraction (RH-1) was found to contain little ß-endorphin-like immunoreactivity or opiate receptor binding activity as shown in figure 4-10 and table 4-2. Examining the displacement curves in figure 4-10 and the opiate receptor binding activity results in table 4-2, the relative potencies of the fractions of rat heart derived from CM-cellulose in the two experiments were comparable, strongly supporting the presence of ß-endorphin-like materials that specifically bind to opiate receptors. (RH-5), the fraction with an especially high potency in opiate receptor assay was found to be also the most potent one in the ß-endorphin RIA, indicating that it was

Table 4-1. Steroidogenic activities of rat heart fractions eluted from CM-cellulose

Fraction	Dose (mg)	Steroidogenic activity (ng corticosterone/hr/25,000 cells)
Control	0	0.011 ± 0.003
ACTH	2 (nM)	0.545 ± 0.085 ^
"	0.567 (nM)	0.015 ± 0.001
RH-1	1	0.237 ± 0.065
"	0.1	0.015 ± 0.003
RH-2	1	0.389 ± 0.092
"	0.1	0.335 ± 0.279
RH-3	0.1	0.057 ± 0.009 ^^
"	0.01	0.018 ± 0.000
RH-4	0.1	0.158 ± 0.014 ^
"	0.01	0.009 ± 0.005
RH-5	0.1	0.203 ± 0.051
"	0.01	0.345 ± 0.275
RH-6	0.1	0.241 ± 0.016 ^
"	0.01	0.018 ± 0.001

Values represent mean ± S.E.M. of triplicate determinations.

^ : $p < 0.005$ compared with control
^^ : $p < 0.01$ compared with control

Table 4-2. Opiate binding activity of rat heart fractions eluted from CM-cellulose

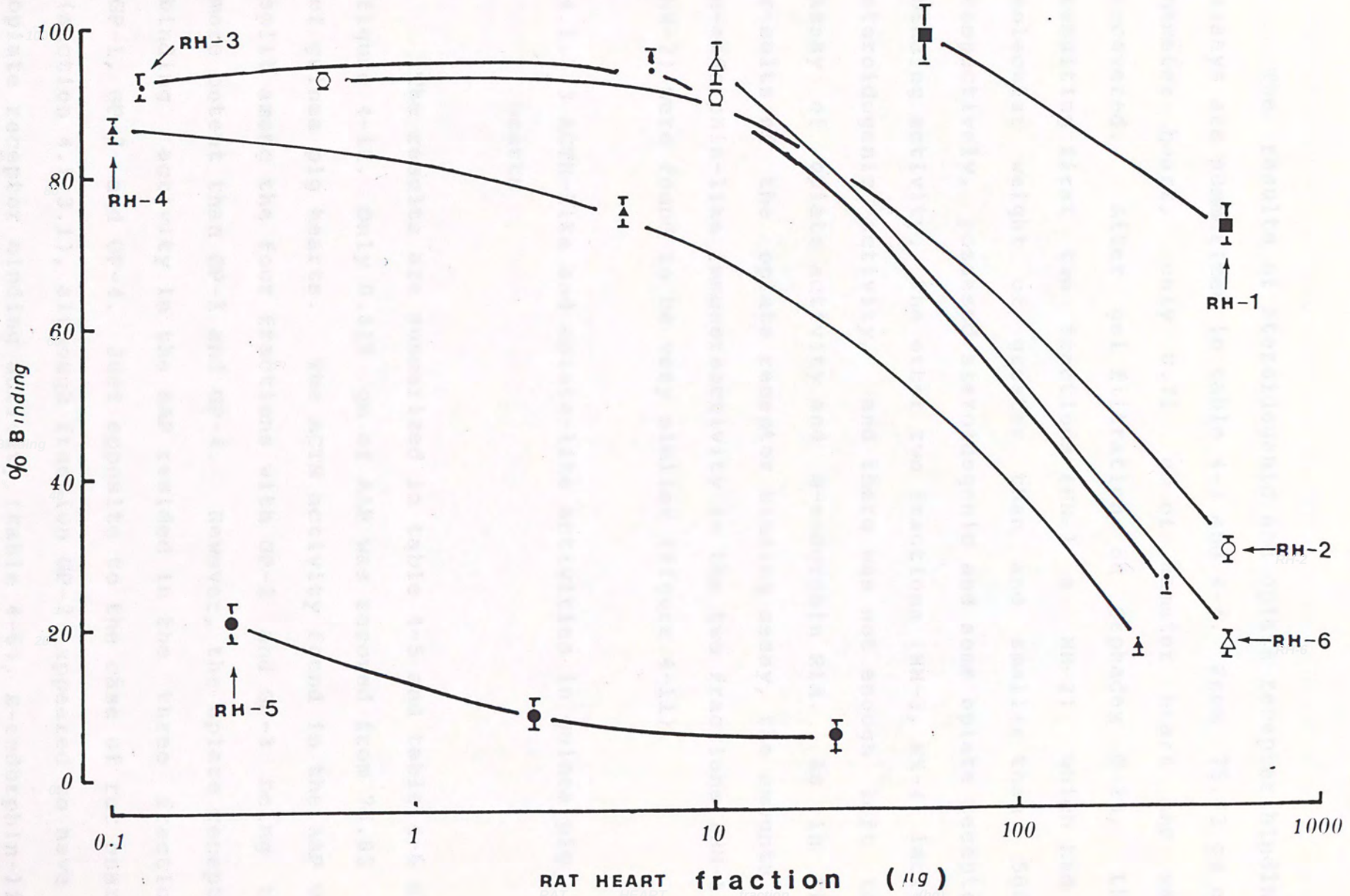
Fraction	Dose (mg)	Opiate receptor binding activity %binding
Leu-enkephalin	0.313 (μg)	22.9 ± 3.14 ^
"	0.078 (μg)	52.0 ± 1.37 ^
"	0.02 (μg)	70.3 ± 0.61 ^
"	0.005 (μg)	80.4 ± 1.58 ^^
RH-1	0.5	82.6 ± 1.33 ^^
"	0.05	91.6 ± 1.89
"	0.005	96.2 ± 2.86
RH-2	0.5	58.5 ± 2.36 ^
"	0.05	93.9 ± 1.14
"	0.005	95.5 ± 1.66
RH-3	0.5	52.1 ± 1.60 ^
"	0.05	94.3 ± 2.93
"	0.005	97.9 ± 1.17
RH-4	0.5	43.3 ± 2.79 ^
"	0.05	91.9 ± 1.01
"	0.005	103.2 ± 1.84
RH-5 (β+)	0.5	23.8 ± 1.67 ^
"	0.05	73.0 ± 1.09 ^
"	0.005	96.8 ± 4.13
RH-6	0.5	57.6 ± 4.57 ^^
"	0.05	96.6 ± 2.72
"	0.005	92.0 ± 2.58

Values represent mean ± S.E.M. of triplicate determinations.

^ : $p < 0.001$ compared with buffer
^^ : $p < 0.005$ compared with buffer
(β+) : The most potent fraction in β-endorphin RIA

Figure 4-10

Displacement of [^{125}I]ß-endorphin from ß-endorphin antiserum by the fractions of CM-cellulose derived from rat hearts


100
80
60
40
20
0
% Binding
RH-3
RH-4
RH-1
RH-2
RH-6
RH-5
0·1
1
10
100
1000
RAT HEART fraction (µg)

riched in β-endorphin-like materials.

4.1.3.2 ACTH-like and opiate-like activities in hamster hearts

The results of steroidogenic and opiate receptor binding assays are summarized in table 4-3 and 4-4. From 76.83 gm of hamster heart, only 0.71 gm of hamster heart AAP was recovered. After gel filtration on Sephadex G-25, the resulting first two fractions (HH-1 & HH-2) which had a molecular weight of greater than and smaller than 5000 respectively, possessd steroidogenic and some opiate receptor binding activity. The other two fractions (HH-3, HH-4) lacked steroidogenic activity, and there was not enough left for assay of opiate activity and β-endorphin RIA. As in the results of the opiate receptor binding assay, the amounts of β-endorphin-like immunoreactivity in the two fractions (HH-1 & HH-2) were found to be very similar (figure 4-11).

4.1.3.3 ACTH-like and opiate-like activities in guinea pig hearts

The results are summarized in table 4-5 and table 4-6 and figure 4-12. Only 0.819 gm of AAP was recoved from 76.83 gm of guinea pig hearts. The ACTH activity found in the AAP was split among the four fractions with GP-2 and GP-3 being the more potent than GP-1 and GP-4. However, the opiate receptor binding activity in the AAP resided in the three fractions GP-1, GP-3 and GP-4. Just opposite to the case of rat hearts (section 4.1.3.1), although fraction GP-2 appeared to have no opiate receptor binding activity (table 4-6), β-endorphin-like

Table 4-3. Steroidogenic activity of hamster heart fractions eluted from Sephadex G-25

Fraction	Dose (mg)	Steroidogenic activity (ng corticosterone/hr/25,000 cells)
Control	0	0.001 ± 0.000
ACTH	1 (nM)	1.433 ± 0.140 ^
"	0.5 (nM)	0.475 ± 0.044 ^
HH-AAP	0.1	0.139 ± 0.014 ^
"	0.01	0.700 ± 0.007 ^
HH-1	0.1	0.328 ± 0.041 ^^
"	0.01	0.031 ± 0.026
"	0.001	UD
HH-2	0.1	0.149 ± 0.129
"	0.01	0.001 ± 0.001
"	0.001	UD
HH-3	2	UD
"	0.2	UD
"	0.02	UD
HH-4	trace	UD

Values represent mean ± S.E.M. of triplicate determinations.

ND : Not determined due to insufficient sample
UD : Undetectable
^ : $p < 0.001$ compared with control
^^ : $p < 0.002$ compared with control

Table 4-4. Opiate binding activity of hamster heart fractions eluted from Sephadex G-25

Fraction	Dose (mg)	Opiate receptor binding activity %binding
Leu-enkephalin	0.078 (μg)	52.0 ± 1.37 ^
"	0.02 (μg)	70.3 ± 0.61 ^
"	0.005 (μg)	80.4 ± 1.58 ^^
MH-1	0.5	72.4 ± 4.51
"	0.05	95.4 ± 2.42
"	0.005	95.0 ± 4.34
MH-2	0.5	72.2 ± 0.59 ^
"	0.05	100.2 ± 2.92
MH-3	trace	ND
MH-4	trace	ND

Values represent mean ± S.E.M. of triplicate determinations.

ND : Not determined due to insufficient sample
^ : $p < 0.001$ compared with buffer
^^ : $p < 0.005$ compared with buffer

Figure 4-11

Displacement of [^{125}I]β-endorphin from β-endorphin antiserum by the fractions of CM-cellulose derived from hamster hearts

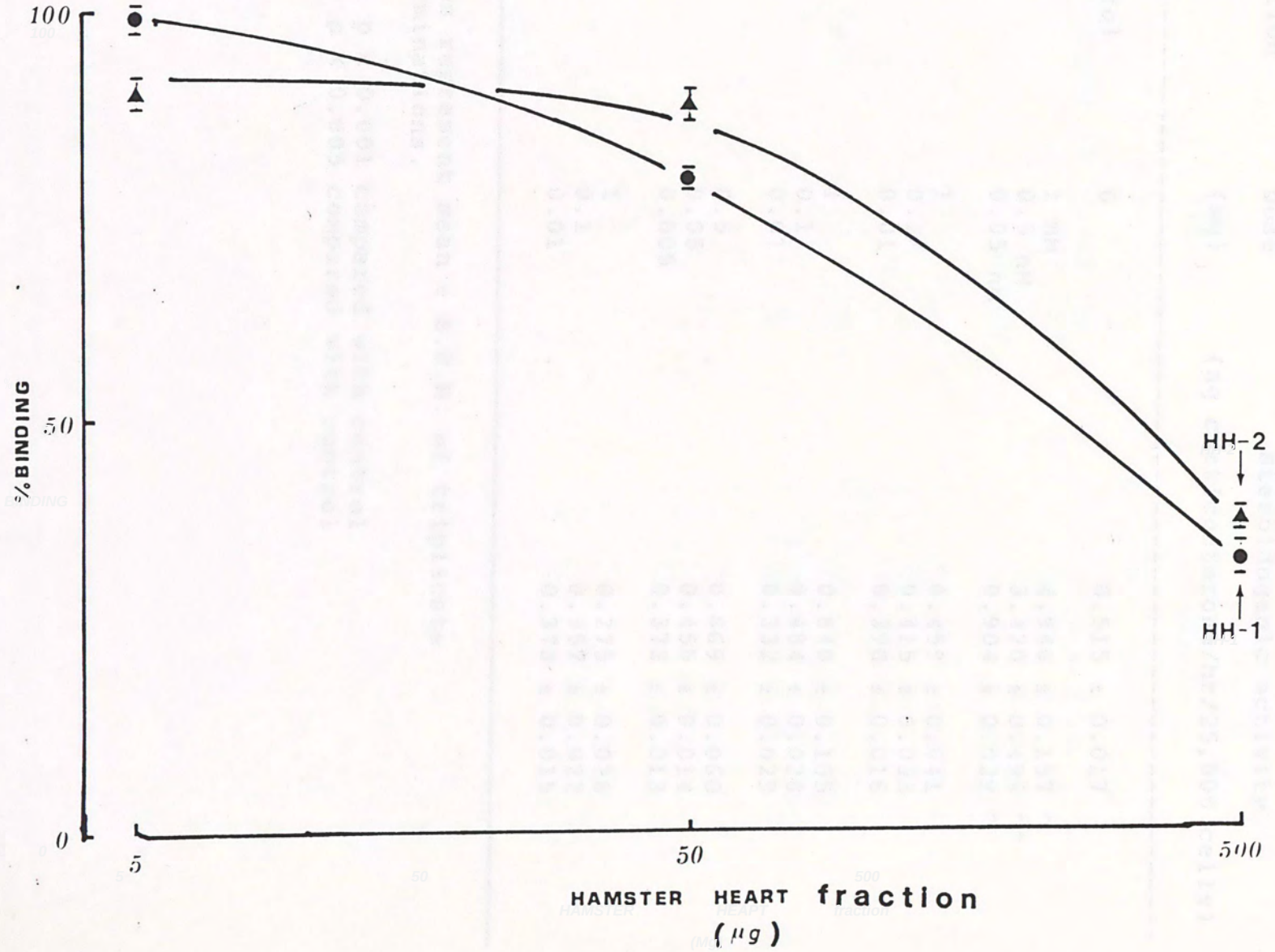

100
50
0
% BINDING
5
50
500
HAMSTER HEART fraction
(μg)
HH-2
HH-1

Table 4-5. Steroidogenic activity of guinea pig heart fractions eluted from Sephadex G-25

Fraction	Dose (mg)	Steroidogenic activity (ng corticosterone/hr/25,000 cells)
Control	0	0.515 ± 0.017
ACTH	1 nM	4.948 ± 0.157 ^
"	0.5 nM	3.420 ± 0.496 ^^
"	0.05 nM	0.904 ± 0.029 ^
GP-1	1	0.459 ± 0.041
"	0.1	0.415 ± 0.033
"	0.01	0.390 ± 0.016
GP-2	1	0.840 ± 0.105
"	0.1	0.484 ± 0.028
"	0.01	0.332 ± 0.029
GP-3	0.5	0.669 ± 0.060
"	0.05	0.455 ± 0.014
"	0.005	0.372 ± 0.018
GP-4	1	0.275 ± 0.028
"	0.1	0.352 ± 0.022
"	0.01	0.373 ± 0.015

Values represent mean ± S.E.M. of triplicate determinations.

^ : $p < 0.001$ compared with control
^^ : $p < 0.005$ compared with control

Table 4-6. Opiate binding activity of guinea pig heart fractions eluted from Sephadex G-25

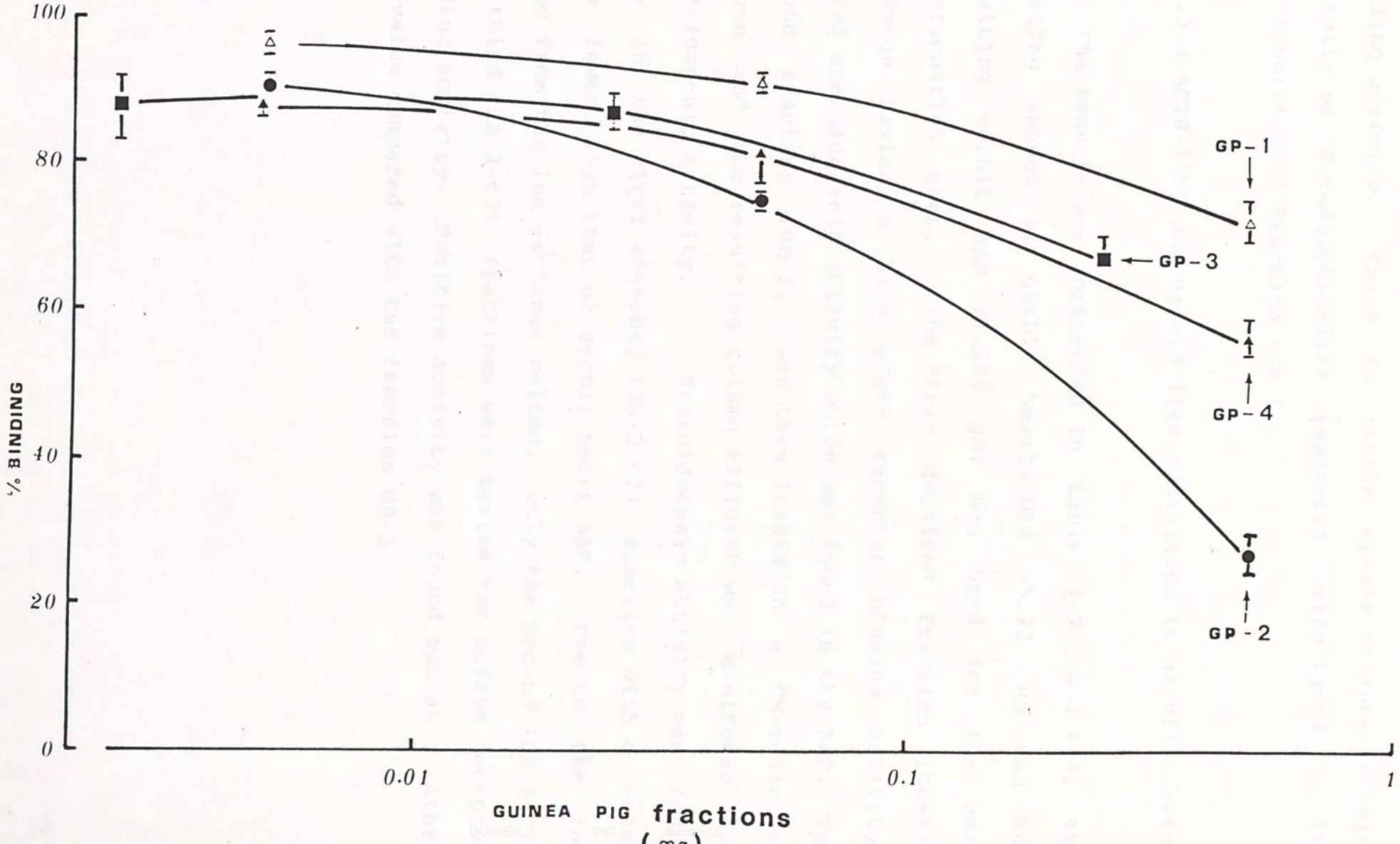
Fraction	Dose (mg)	Opiate receptor binding activity %binding
Leu-enkephalin	0.313 (µg)	22.9 ± 3.14 ^
"	0.078 (µg)	52.0 ± 1.37 ^
"	0.02 (µg)	70.3 ± 0.61 ^
"	0.005 (µg)	90.4 ± 1.58 ^^
GP-1	0.5	85.2 ± 1.93
"	0.05	88.7 ± 2.51
"	0.005	101.5 ± 2.24
GP-2 (ß+)	0.5	93.2 ± 4.41
"	0.05	104.6 ± 2.14
"	0.005	99.0 ± 3.27
GP-3	0.5	75.2 ± 2.78 ^^
"	0.05	94.1 ± 0.78
"	0.005	93.7 ± 1.56
GP-4	0.5	46.9 ± 3.37 ^
"	0.05	82.8 ± 1.21 ^^
"	0.005	97.0 ± 2.29

Values represent mean ± S.E.M. of triplicate determinations.

^ : $p < 0.001$ compared with buffer
^^ : $p < 0.005$ compared with buffer
(ß+) : The most potent fraction in ß-endorphin RIA

Figure 4-12

Displacement of [^{125}I]ß-endorphin from ß-endorphin antiserum by the fractions of CM-cellulose derived from guinea pig hearts


100
80
60
40
20
0
% BINDING
0.01
0.1
1
GUINEA PIG fractions
(mg)
GP-1
GP-3
GP-4
GP-2

immunoreactivity was found to be the most potent in this fraction (figure 4-12). Fractions GP-3 and GP-4 possessed both β-endorphin-like immunoreactivity and opiate receptor binding activity. There is little opiate receptor binding activity or β-endorphin-like immunoreactivity found in the first unretarded fraction (GP-1).

4.1.3.4 ACTH-like and opiate-like activities in gerbil hearts

The results are presented in table 4-7 and 4-8. The starting amount of gerbil hearts was 95.72 gm and the resulting gerbil AAP (1.186 gm) was used for the salt fractionation step. The first desalted fraction (GB-1), although having a high opiate receptor binding activity, lacked steroidogenic activity which was found in the AAP. The second fraction (GB-2) was then loaded on a CM-cellulose column and the resulting column effluent was monitored for steroidogenic activity. Steroidogenic activity was found only in the first adsorbed (GB-2-C2) fraction with a value even lower than that of gerbil heart AAP. Due to the low yield from the ion-exchange column, only the second (GB-2-C2) and third (GB-2-C3) fractions were tested for opiate receptor binding activity. Positive activity was found but at a rather low value compared with the fraction GB-1.

Table 4-7. Steroidogenic activity of gerbil heart fractions eluted from Sephadex G-10 and CM-cellulose

Fraction	Dose (mg)	Steroidogenic activity (ng corticosterone/hr/25,000 cells)
Control	0	0.001 ± 0.000
ACTH	1 (nM)	1.433 ± 0.140 ^
"	5 (nM)	0.475 ± 0.044 ^
GB-AAP	1 mg	0.499 ± 0.073 ^^
GB-1	0.1	UD
"	0.01	0.005 ± 0.001
"	0.001	0.001 ± 0.000
GB-2-C1	trace	UD
GB-2-C2	1	0.064 ± 0.036
"	0.1	0.002 ± 0.000
"	0.01	0.006 ± 0.004
GB-2-C3	1	0.007 ± 0.005
"	0.1	UD
"	0.01	UD
GB-2-C4	trace	UD

Values represent mean ± S.E.M. of triplicate determinations.

^ : $p < 0.001$ compared with control
^^ : $p < 0.005$ compared with control

Table 4-8. Opiate binding activity of gerbil heart fractions eluted from Sephadex G-10 and CM-cellulose

Fractoins	Experiment 1		Experiment 2	
	Dose	Opiate receptor binding activity	Dose	Opiate receptor binding activity
	(mg)	%Binding	(mg)	%Binding
LEU-enk	0.046 (μg)	19.5 ± 1.27 ^	0.014 (μg)	14.7 ± 1.34 ^
"	0.015 (μg)	29.9 ± 9.78 ^^	0.005 (μg)	35.2 ± 0.33 ^
GB-1	0.5	19.4 ± 5.07 ^	-	-
"	0.05	51.6 ± 0.39 ^	-	-
"	0.005	73.1 ± 0.85 ^	-	-
GB-2-C1	-	-	trace	ND
GB-2-C2	-	-	0.5	71.1 ± 2.21 ^
"	-	-	0.05	100.0 ± 0.49
"	-	-	0.005	99.2 ± 2.12
GB-2-C3	-	-	0.5	72.3 ± 3.41 ^^
"	-	-	0.05	96.5 ± 2.67
"	-	-	0.005	95.9 ± 3.39
GB-2-C4	-	-	trace	ND

Values represent mean ± S.E.M. of triplicate determinations.

ND : Not determined due to insufficient sample

^ : $p < 0.001$ compared with buffer
^^ : $p < 0.005$ compared with buffer

4.1.4 Discussion - mammalian hearts

4.1.4.1 About the various steps in the purification scheme

Various methods such as ion exchange chromatography, gel filtration and salt fractionation were applied to purify the hormones from different species. In the case of rat hearts, CM-cellulose chromatography seems to provide good purification results although there was a relatively low recovery (figure 4-2). Although sodium ion does not have a significant effect on k receptor (Cheney and Lahti, 1987), it also gives a displacement curve in the opiate receptor binding assay (Werling et al, 1986). However, it is not adsorbed on CM-cellulose at low pH. That little opiate-like activity was found in the unadsorbed fraction from CM-cellulose seems to support the idea that any sodium ions present in the original tissue was removed by the acid acetone extraction method which was applied throughout this thesis.

In inclusion of the salt fractionation step in the purification scheme resulted in some loss of ACTH-like activity in the vsdr of gerbil heart (table 4-7). As a comparison, the chromatography of guinea pig and hamster heart extracts on Sephadex G-25 resulted in much better recoveries and some additional information on the molecular weight of the active material.

4.1.4.2 About the findings from rodent hearts

The results indicate that the presence of ACTH-like and opiate-like activities seems to be universal to the heart tissues of the rodents studied. This finding is consistent with the previous studies on other rodent species in which the identification of the opiate was based mainly on HPLC and RIA evidence (section 4.1.1). However, the nature of the opiate-like materials found in the guinea pig heart fractions were believed to be not the same of that found in rat heart fractions since the opiate containing fraction of rat heart possess highest β-endorphin immunoreactivity while the highest β-endorhpin immunoreactivity fraction of guinea heart was found to be totally inactive in the opiate receptor binding assay.

ACTH-like material with a molecular weight greater than and smaller than 5000 were found in the hamster heart fractions, HH-1 & HH-2, respectively. Furthermore, high and low molecular weight materials with opiate activity were also found (table 4-4; figure 4-11) and thus suggesting the possibility of the coexistence of precursor-like material of opiate and ACTH in hamster heart.

Presence of low molecular weight opiate-like materials in the guinea pig hearts may indicate the presence of small opioid peptides such as enkephalin which had previously been reported in guinea pig heart (Lang et al, 1983) but a positive result in the β-endorphin RIA (figure 4-11) also imply the presence of β-endorphin-related peptide. The unretarded peak from the Sephadex G-25 colulmn (MW > 5,000) possessed only low opiat-like activity.

4.1.4.3 About the implications of the findings and perspectives

The actual physiological functions of ACTH and opiate in the heart are still not well-known. It seems that ACTH causes hypertension (McDongall et al, 1980) with an indirect mechanism (Biglieri and Kater, 1983) that may involve the production of prostaglandins (Mason et al, 1984). On the other hand, endogenous opioid peptides were reported to have both pressor and depressor effects on blood pressure depending on the experimental conditions and the type of opiate receptors involved (Holaday, 1983). It is now believed that the actions of endogenous opiates are mainly hypotensive (Akil, 1984) although the actual mechanism may be rather complicated due to the intrinsic complexity of the endogenous opiate system in the body. An indirect mechanism through the authonomic nervous system by the μ-receptor (Kiritsy-Roy et al, 1986) of endogenous opiates on the cardiovascular system was suggested. Although the cardiodepressant actions of met-enkephalin was believed to be exerted on the central nervous system (Eulie and Rhee, 1984) the discovery that β-endorphin reduced both left ventricular systolic and diastolic pressures (Wong et al, 1985) of the isolated rat heart also suggested a possible direct mechanism of local opiate and/or POMC-derived peptides on the heart. The finding of materials with opiate-like activity in the heart thus adds significance to the aforementioned observation.

The idea that the heart may function as an endocrine

gland in the body (Genest, 1985) began with the discovery of a family of peptides called atrial natriuretic factor (ANF) (Atlas, 1986). ANF has potent natriuretic, diuretic, and relaxant activities and is released from atrial cardiocytes (Genest, 1985). Recently, γ-MSH, a peptide derived from the N-terminal of POMC (the common precursor of ACTH and β-endorphin), was found to share many properties of ANF (Gruber et al, 1985) and thus a possible relationship between POMC-derived peptides and the heart functions was suspected. However, the ACTH-like material is probably not ANF since although ANF has been found to affect the secretion of aldosterone from the outer zone of adrenal cortex, it has no effect on corticosterone production by isolated rat fasciculata-reticularis cells (Atarashi et al, 1984). Whether the materials found in hamster hearts bear any relationship to POMC and to ANF remains to be elucidated. Furthermore, some of these factors may activate vagal afferents and thus may alter pain perception (Thoren et al, 1985), indicating a possible relation of ANF and the opiate activity found in the hearts.

4.2 Mammalian testes

4.2.1 Introduction - ACTH and opiates in mammalian testes

It was recently found that ACTH directly and specifically stimulated testosterone secretion by rabbit testes perfused *in vitro* (Juniewice et al, 1986). Whether the physiological significance of this direct action of ACTH on the testes is related to testicular ACTH (if there is any) or to ACTH of extra-testicular (such as pituitary) origin remains a question. However, the identification of POMC-derived peptides (Bardin et al, 1984) and mRNA-like activity in the rat testes (Chen et al, 1984; Pintar et al, 1984) suggests a possible local source of ACTH and β-endorphin in the testes.

Unlike ACTH, β-endorphin-like materials have already been found in the Leydig cells of testes of rodents such as the mouse, hamster and guinea pig (Tsong et al, 1982) using immunocytochemical methods and the rat (Margioris et al, 1983) using radioimmunoassay (RIA). Thus, a role of testicular opiates in the regulation of testicular Leydig cell function in testes was suggested (Gerendai et al, 1984).

The aim of the present investigation was to see if ACTH- and opiate-like materials were also present in animals other than laboratory rodents. Thus the bovine testis was chosen as an example. Furthermore, only HPLC, RIA and immunocytochemical evidences were provided by previous

studies. The present investigation was initiated to examine the existence of materials with biological and radioreceptor binding activities in bovine testes.

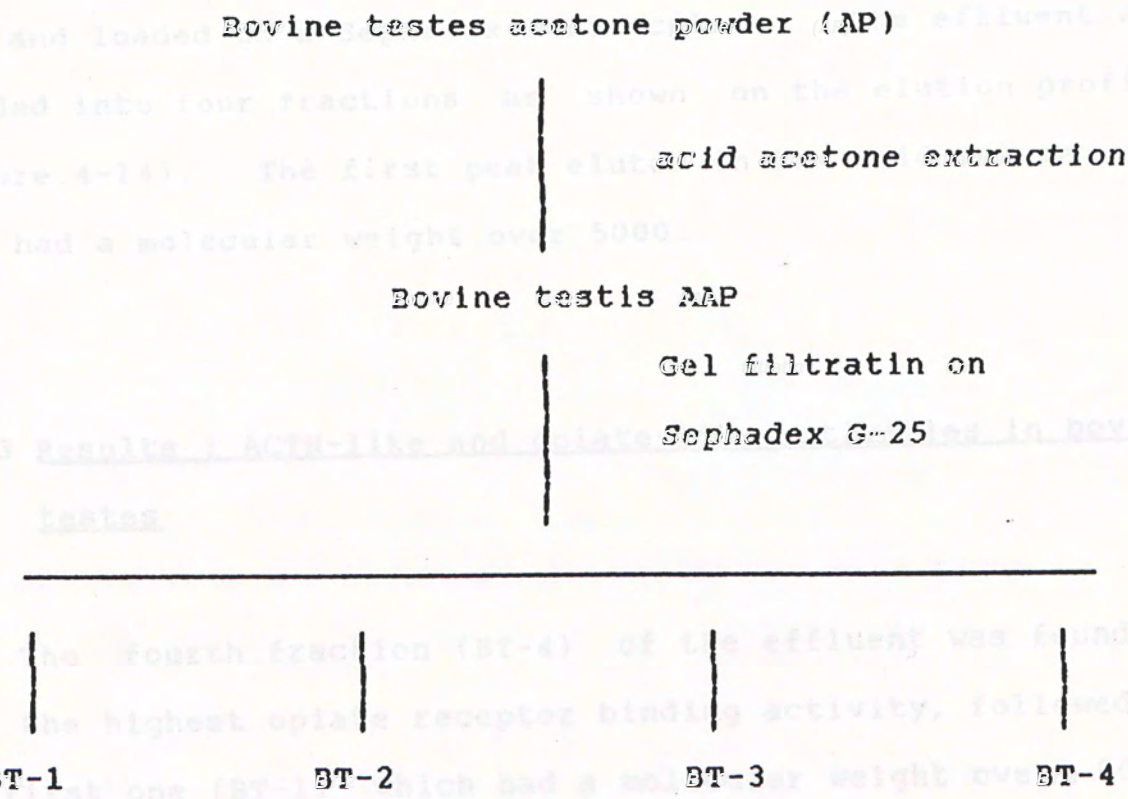
4.2.2 Materials and method - extraction of bovine testes

The protocol for the extraction of bovine testes is shown in figure 4-13. Acetone powder (AP) of bovine testes (Sigma) was used for the preparation of acid acetone powder (AAP). The resulting AAP was subjected to gel filtration on a Sephadex G-25 column.

4.2.2.1 Preparation of bovine testis AAP

Acetone powder (AP) of bovine testes was purchased from Sigma. It was first heated in 5 vol of a mixture of water : HCl (21 : 1) at 90 °C for 20 minutes. Then 9 ml acetone was added and the mixture was homogenized with a Polytron homogenizer (Setting 4, 15 sec) when kept in ice. The homogenate was then centrifuged at 15,000 g for 30 minutes. The supernatant was saved and the pellet was rehomogenized in another 5 volumes (w/v) of 80% acetone when kept in ice. The supernatants from the two centrifugation steps were pooled. The final mixture was allowed to stand at 4 °C for 24 hours and the precipitate was collected on filter paper (Whatman no. 1). The precipitate was then washed with 50 ml of cold acetone and lyophilized. The lyophilized material, i.e. the acid acetone powder (AAP) of bovine testes, was kept in -20 °C until further purification.

Figure 4-13 Extraction and purification of ACTH-like and opiate-like activities from bovine testes



4.2.2.2 Chromatographic purification on Sephadex G-25

The AAP of bovine testes was dissolved in 0.1 N acetic acid and loaded on a Sephadex G-25 column. The effluent was divided into four fractions as shown on the elution profile (figure 4-14). The first peak eluted in the void volume and thus had a molecular weight over 5000.

4.2.3 Results : ACTH-like and opiate-like activities in bovine testes

The fourth fraction (BT-4) of the effluent was found to have the highest opiate receptor binding activity, followed by the first one (BT-1) which had a molecular weight over 5000. The second one (BT-2) was inactive while the yield of the third fraction (BT-3) was too low to permit assay (figure 4-15). Steroidogenic activity was also found in the AAP of bovine testes (table 4-9)

Figure 4-14

Gel filtration of bovine testis AAP (209 mg) on a Sephadex G-25 column (3.5 x 77 cm). Fraction size = 5 ml.

Buffer: 0.1 M acetic acid.

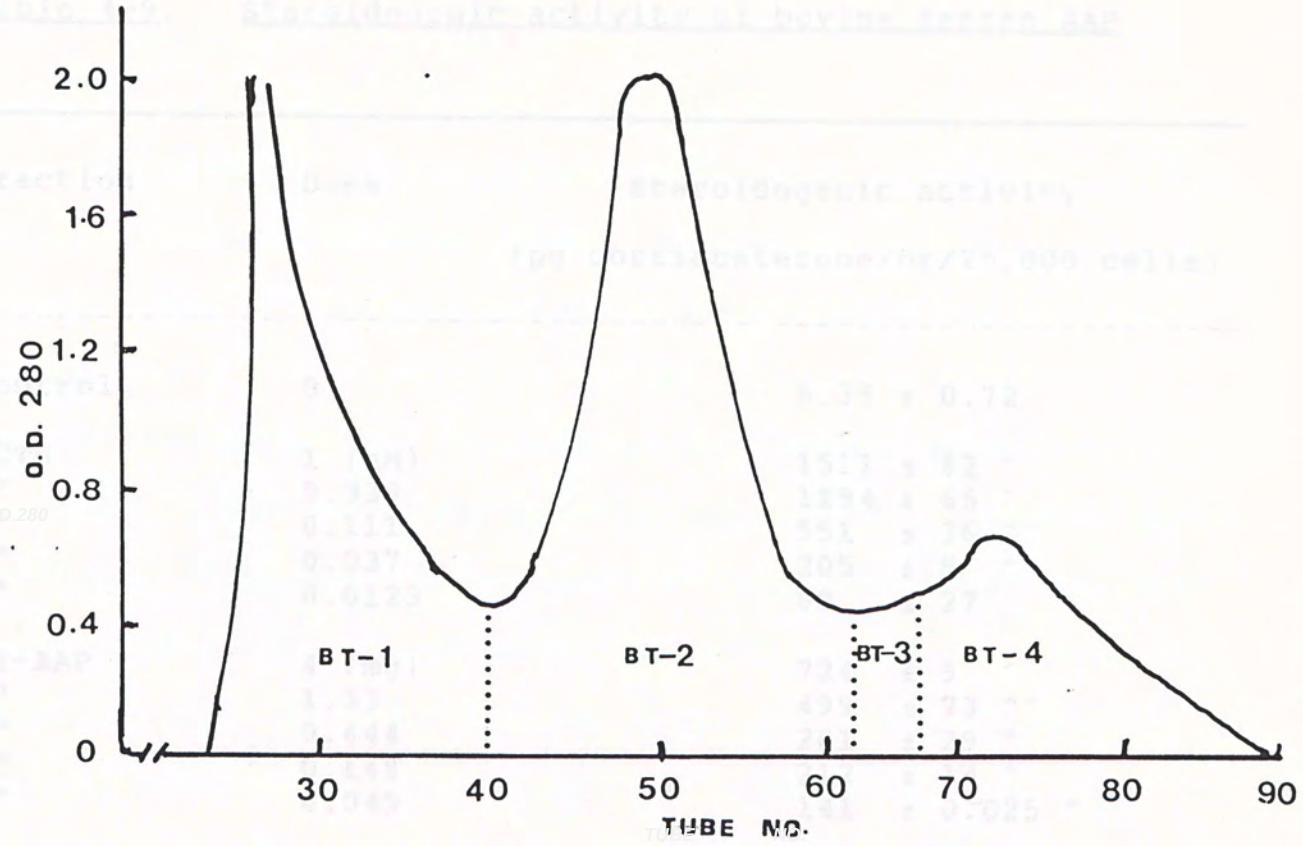
Yields: BT-1, 15.5 mg; (Void volume)
BT-2, 100 mg;
BT-3, 3 mg;
BT-4, 53 mg.

Figure 4-15

Opiate radioreceptor binding assay data of Sephadex G-25 fractions derived from bovine testis AAP :

Standard: LEK (Leucine enkephalin)

Ligand: DADLE


2.0
1.6
1.2
0.8
0.4
0
O.D. 280
BT-1
BT-2
BT-3
BT-4
30
40
50
60
70
80
90
TUBE NO.

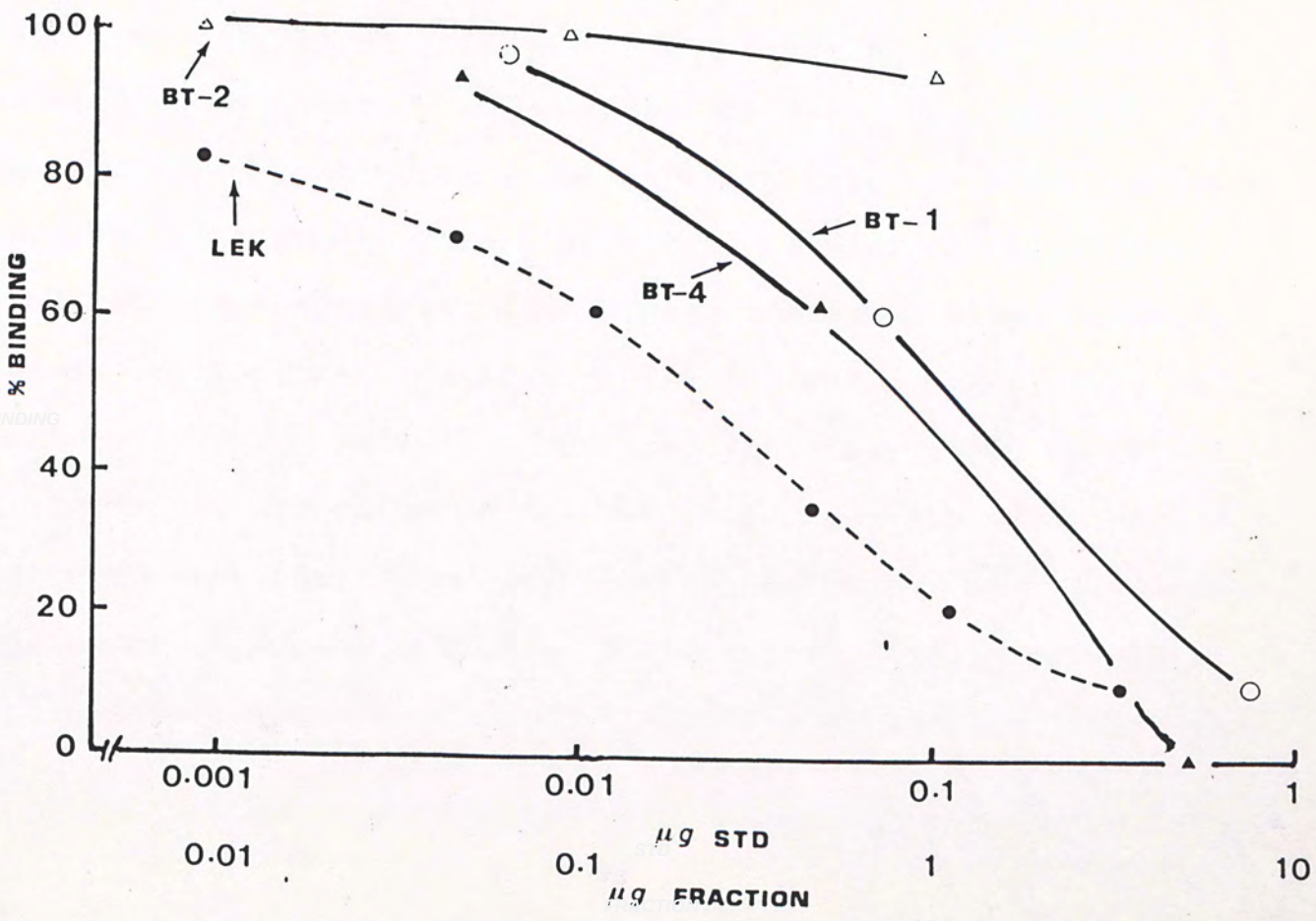

100
80
60
40
20
0
% BINDING
BT-2
LEK
BT-1
BT-4
0.001
0.01
0.1
1
μg STD
0.01
0.1
1
10
μg FRACTION

Table 4-9. Steroidogenic activity of bovine testes AAP

Fraction	Dose	Steroidogenic activity (pg corticosterone/hr/25,000 cells)
Control	0	5.35 ± 0.72
ACTH	1 (nM)	1511 ± 92 ^
"	0.333	1154 ± 65 ^
"	0.111	551 ± 36 ^
"	0.037	205 ± 5 ^
"	0.0123	80 ± 27
BT-AAP	4 (mg)	724 ± 9 ^
"	1.33	499 ± 73 ^^
"	0.444	281 ± 29 ^
"	0.148	212 ± 14 ^
"	0.049	141 ± 0.025 ^

Values represent mean ± S.E.M. of triplicate determinations.

^ : $p < 0.001$ compared with control
^ : $p < 0.005$ compared with control

4.2.4 Discussion - bovine testes

The presence of endogenous opiates in the testis has been reported only in several rodents (Tsong et al, 1982; Margioris et al, 1983). The present study demonstrates the presence of opiate receptor binding activity in the testis of a large mammal (bovine). The presence of high opiate receptor binding activity in both high (BT-1) and low (BT-4) molecular weight fractions suggested the presence of different types of opiate-like materials. Whether these materials have any relationships remains to be studied. The presence of steroidogenic activity in AAP of bovine testes was also demonstrated although the steroidogenic activity of the fractions derived from it was not tested due to lack of material.

The physiological role of opiates and ACTH in regulating testicular functions was not well-known. Although ACTH was found to suppress testosterone release when used at a pharmacological dose (Yrvine et al, 1974), the results of other groups suggested a stimulatory role of ACTH in the secretion of testosterone in male rats (Armario et al, 1986), fetal rats (Warren et al, 1984), pigs (Juniewicz and Johnson, 1984a), rabbits (Pitzel et al, 1984) and boar (Liptrap and Raeside, 1975). Luteinzing hormone (LH) was found to be not involved in this stimulation but the contribution of an indirect mechanism through corticosteroids was suggested (Juniewice and John, 1984a,b; Warren et al, 1984; Armario et al, 1986). However, the stimulation of ACTH on androgen

release in the male rabbit was found to be not mediated by cortisol (Fenske, 1980). Furthermore, it was recently found that ACTH stimulated testosterone secretion directly and specifically by perfused rabbit testes *in vitro* and that there was no synergism between ACTH and LH in this process (Juniewice et al, 1986). In contrast, ACTH failed to produce a similar stimulation of rat testes in the same experiment. Further considering the observation that ACTH failed to stimulate testosterone production in the gerbil (Fenske, 1984), the action of ACTH on testes seems to vary from species to species in the mammals. Hence the physiological role of ACTH-like activity found here in the bovine testes remains to be elucidated.

On the other hand, the actions of endogenous peptides on the reproductive system have usually been attributed to their profound inhibitory effects on LH secretion (Briski et al, 1984; Petraglia et al, 1984; Bernasconi et al, 1986) through the central nervous system and under the influence of gonadal steroids. However, β-endorphin-like materials were found in the Leydig cells of the testes of rodents such as the mouse, hamster, guinea pig and rabbit (Tsong et al, 1982) using immunocytochemical methods and those of the rat (Margioris et al, 1983) using RIA. Thus a role of testicular opiates in the regulation of Leydig cell function in testes was suggested (Gerendai et al, 1984). Furthermore, the discovery of β-endorphin and the two enkephalins in human semen (Fraioli et al, 1984) also suggests a local role of endogenous opioid peptides in the male reproductive systems. Whether the opiate activity found here in the bovine testes plays a similar role

should be studied in the future. The identification of mRNA of POMC in testes (Chen et al, 1984; Pintar et al, 1984) suggests a possible local biosynthesis of ACTH and β-endorphin. Although opiates and ACTH may play a paracrine role in the testes, the possibility of ACTH being a by-product of the biosynthesis of β-endorphin from POMC cannot be simply ruled out before the actual significance of ACTH in the bovine testes is revealed.

4.3 Mammalian pancreas

4.3.1 Introduction - opiates in mammalian pancreas

The existence of β-endorphin (Bruni et al, 1979; Watkins et al, 1980; Houck et al, 1981; Tung and Cockburn, 1984), enkephalin (Stein et al, 1982; Alumets et al, 1983) in the pancreas of various mammalian species including rat, human, guinea pig, porcine, fetal bovine, has already been reported. Dynorphin-like immunoreactivity was also reported in rat pancreas (Spampinato and Goldstein, 1983). In this investigation, the ovine pancreas was studied to see whether the existence of these hormones is universal to mammalian pancreas.

4.3.2 Materials and methods - extraction of ovine pancreas

AAP was prepared from the acetone powder (AP) of ovine pancreas using the method described for bovine testes (section 4.2.2.1). After salt fractionation (section 4.1.2.3b), the material was desalted on a Sephadex G-10 column and results in five fractions (OP-1, OP-2, OP-3, OP-4, OP-5). The unretarded fraction (OP-1) was loaded on Sephadex G-25 and the first resulting fraction (OP-1-1) was then chromatographed on CM-cellulose using a combined ionic and pH gradient. The protocol is shown in figure 4-16. The chromatograms involving the use of G-10, G-25, CM-cellulose were shown in figure 4-17, 4-18, 4-19 respectively.

Figure 4-16 Extraction and purification of ACTH-like and opiate-like activities from ovine pancreas

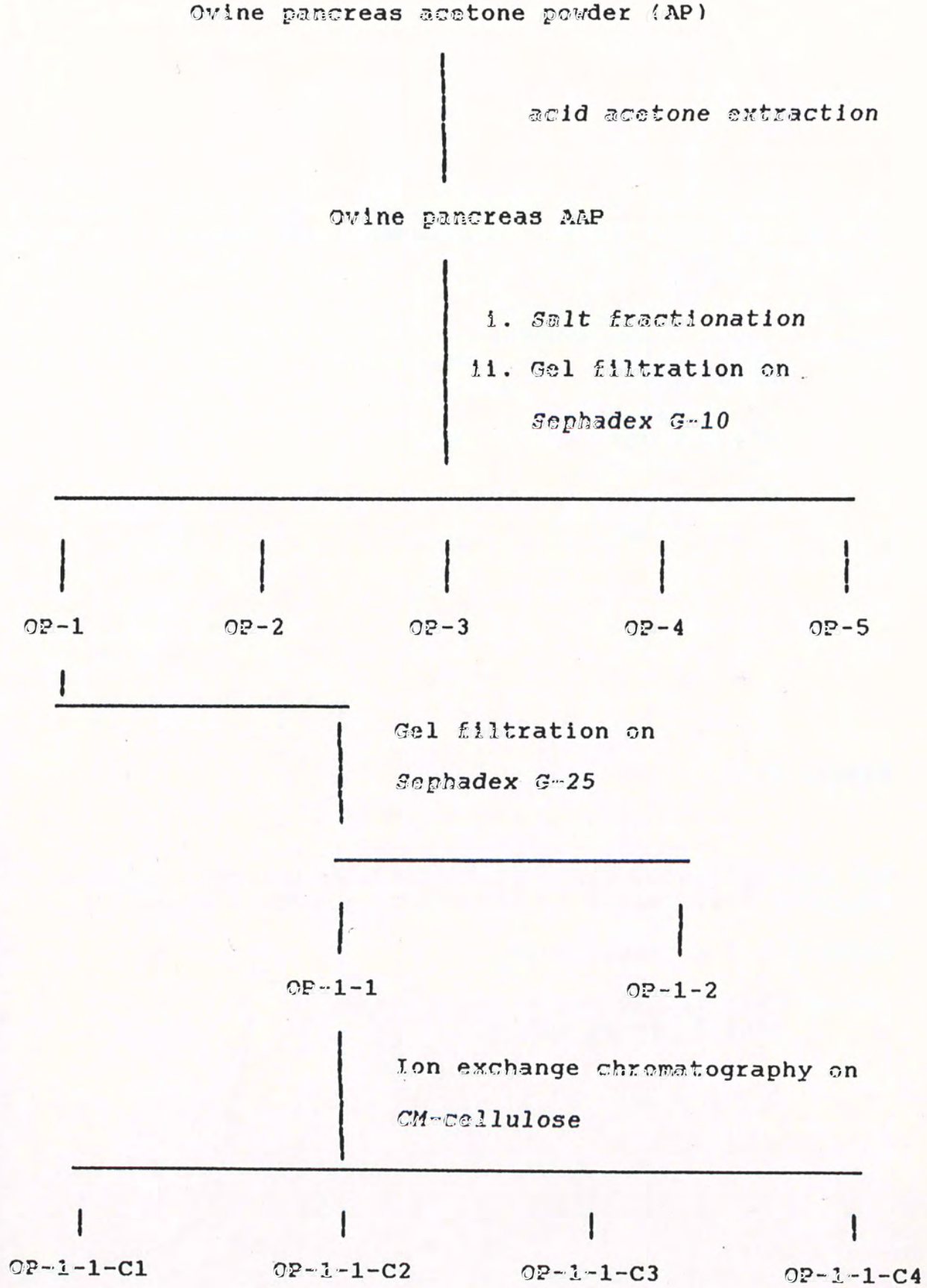


Figure 4-17

Gel filtration of fraction derived from ovine pancreas MAP after salt fraction on a Sephadex G-10 column (2.5 x 78 cm). Fraction size = 5.5 ml.

Buffer: 0.1 M acetic acid.

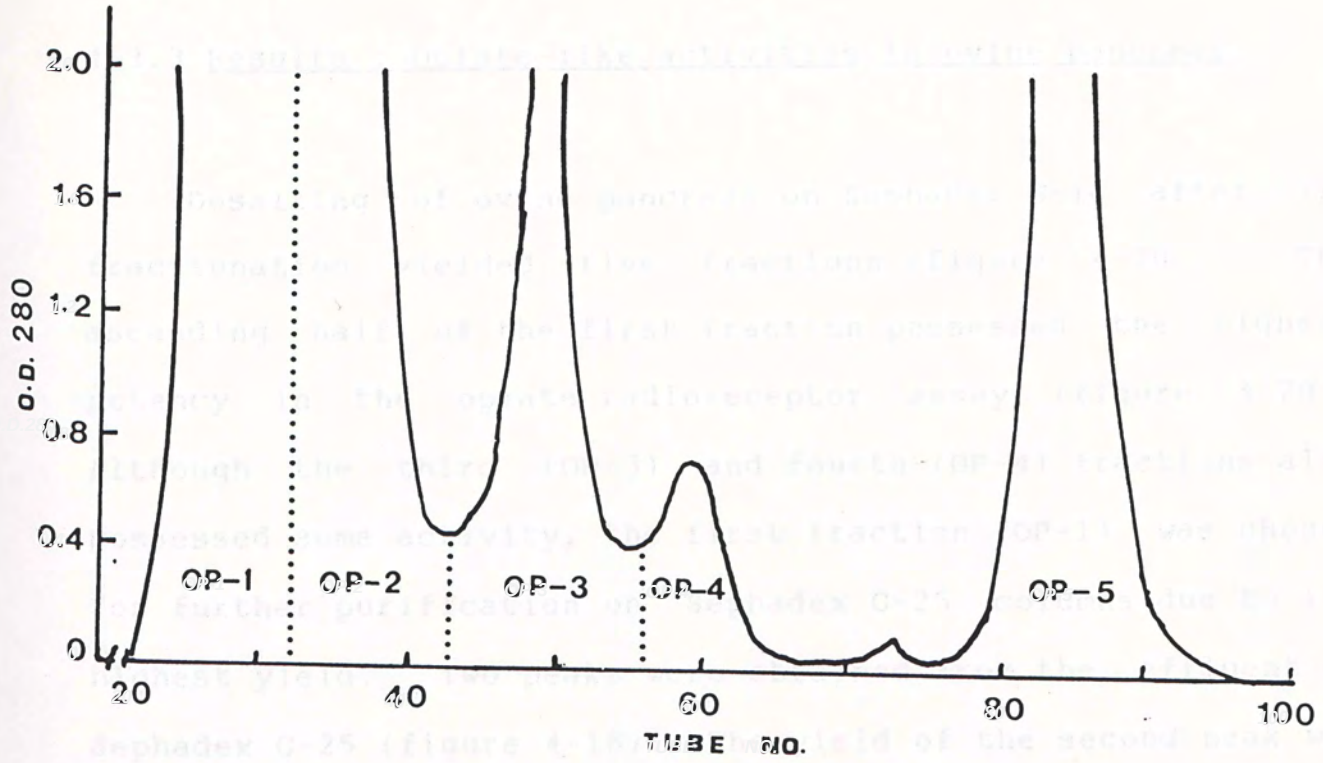
Yield: OP-1, 207 mg; (Void volume)
OP-2, salty;
OP-3, 317 mg;
OP-4, 6 mg;
OP-5, salty.

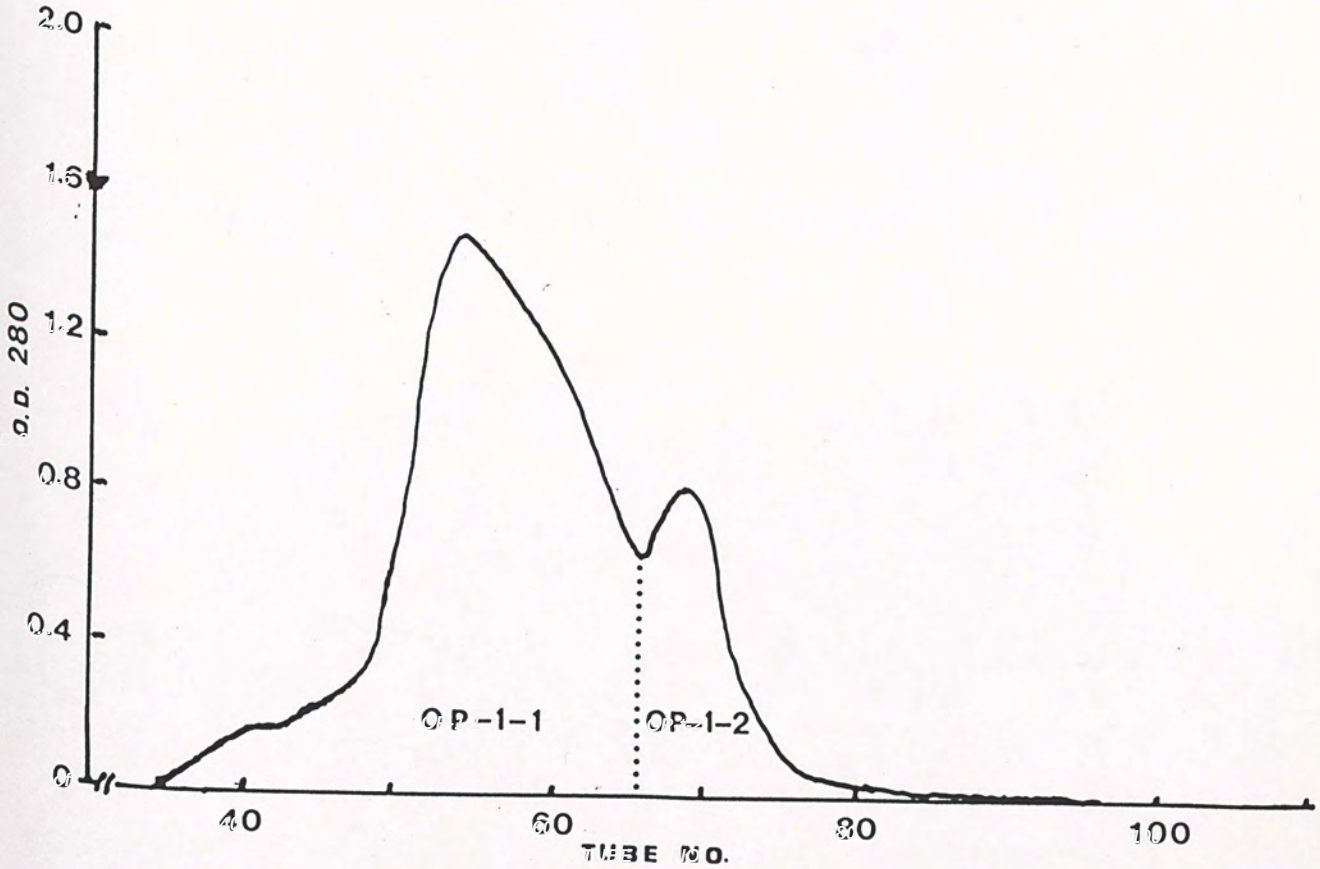
Figure 4-18

Gel filtration of ovine pancreas fraction OP-1 (200 mg) on a Sephadex G-25 column (3 x 80 cm). Fraction size: 7 ml.

Buffer: 0.1 M acetic acid.

Yield: OP-1-1, 143 mg; (Void volume)
OP-1-2, trace amount.


2.0
1.6
1.2
0.8
0.4
0
O.D. 280
OP-1
OP-2
OP-3
OP-4
OP-5
20
40
60
80
100
TUBE NO.


2.0
1.6
1.2
0.8
0.4
0
O.D. 280
OP-1-1
OP-1-2
40
60
80
100
TUBE NO.

4.3.3 Results : Opiate-like activities in ovine pancreas

Desalting of ovine pancreas on Sephadex G-10 after salt fractionation yielded five fractions (figure 4-20). The ascending half of the first fraction possessed the highest potency in the opiate radioreceptor assay (figure 4-20). Although the third (OP-3) and fourth (OP-4) fractions also possessed some activity, the first fraction (OP-1) was chosen for further purification on Sephadex G-25 columns due to its highest yield. Two peaks were obtained from the effluent of Sephadex G-25 (figure 4-18). The yield of the second peak was low and so the first peak (OP-1-1) was taken and chromatographed on CM-celulose. The elution profile is shown in figure 4-19. The resulting fourth fraction (OP-1-1-C4) had the highest receptor binding potency while the other three fractions were by comparison much less active (figure 4-21).

Figure 4-19

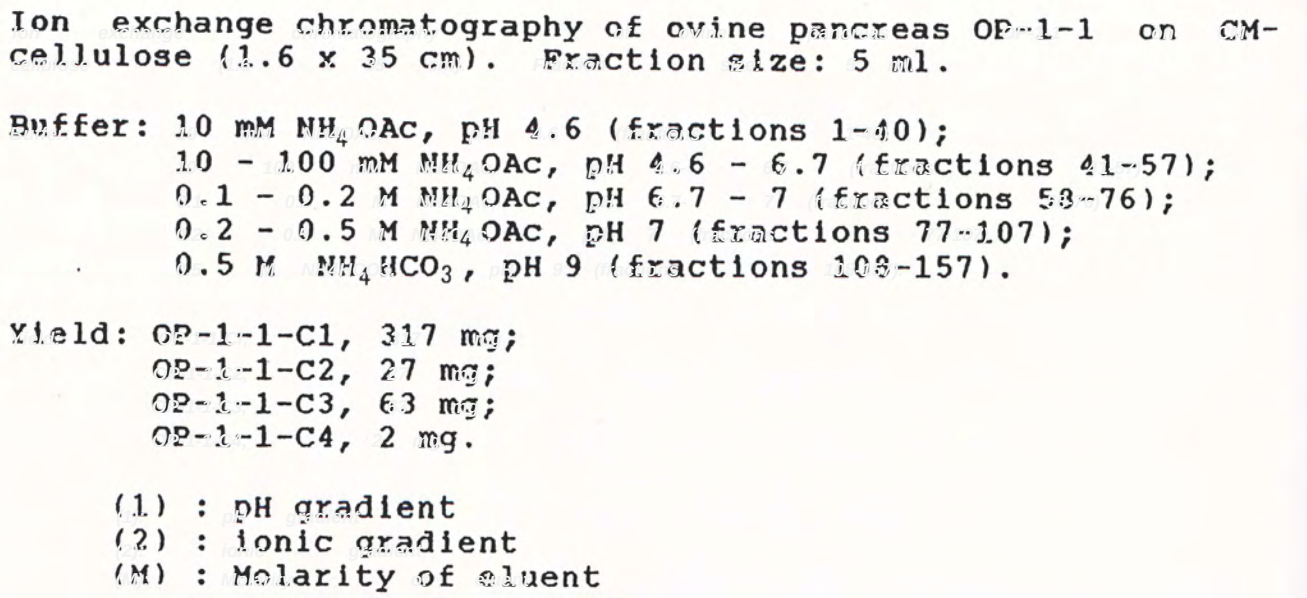
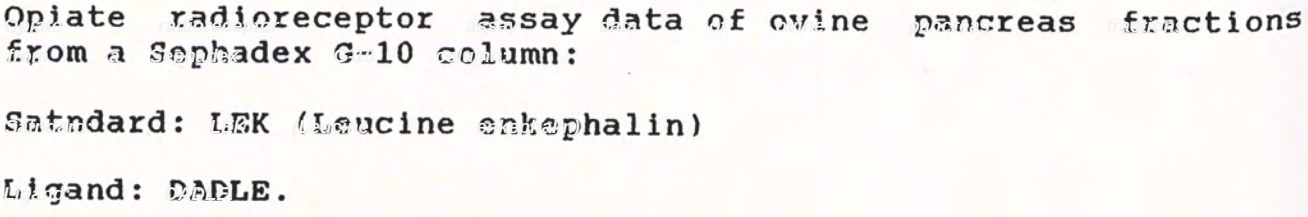
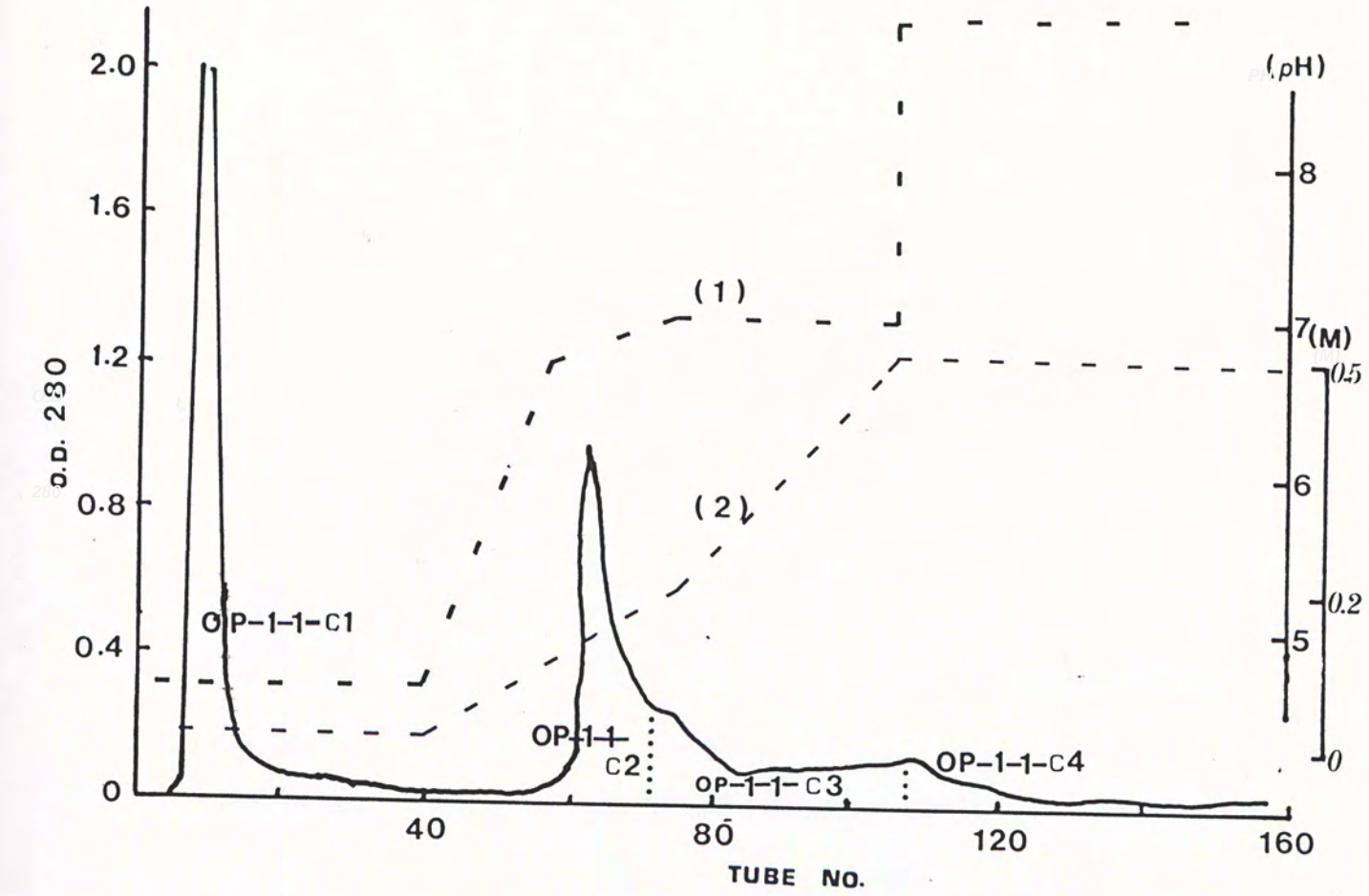


Figure 4-20




O.D. 230
(pH)
(M)
(1)
(2)
OP-1-1-C1
OP-1-1-C2
OP-1-1-C3
OP-1-1-C4
TUBE NO.

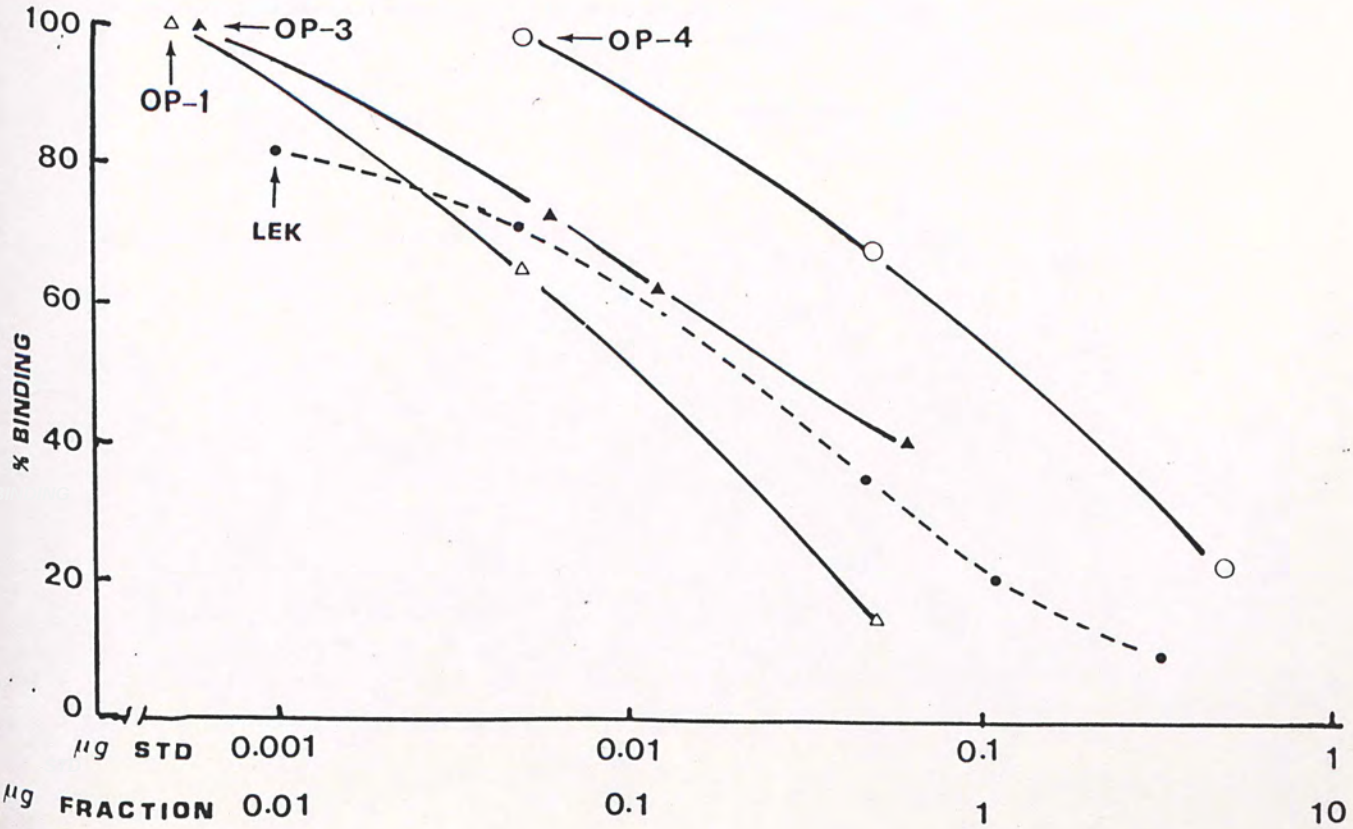

OP-3
OP-4
OP-1
LEK
% BINDING
μg STD
μg FRACTION

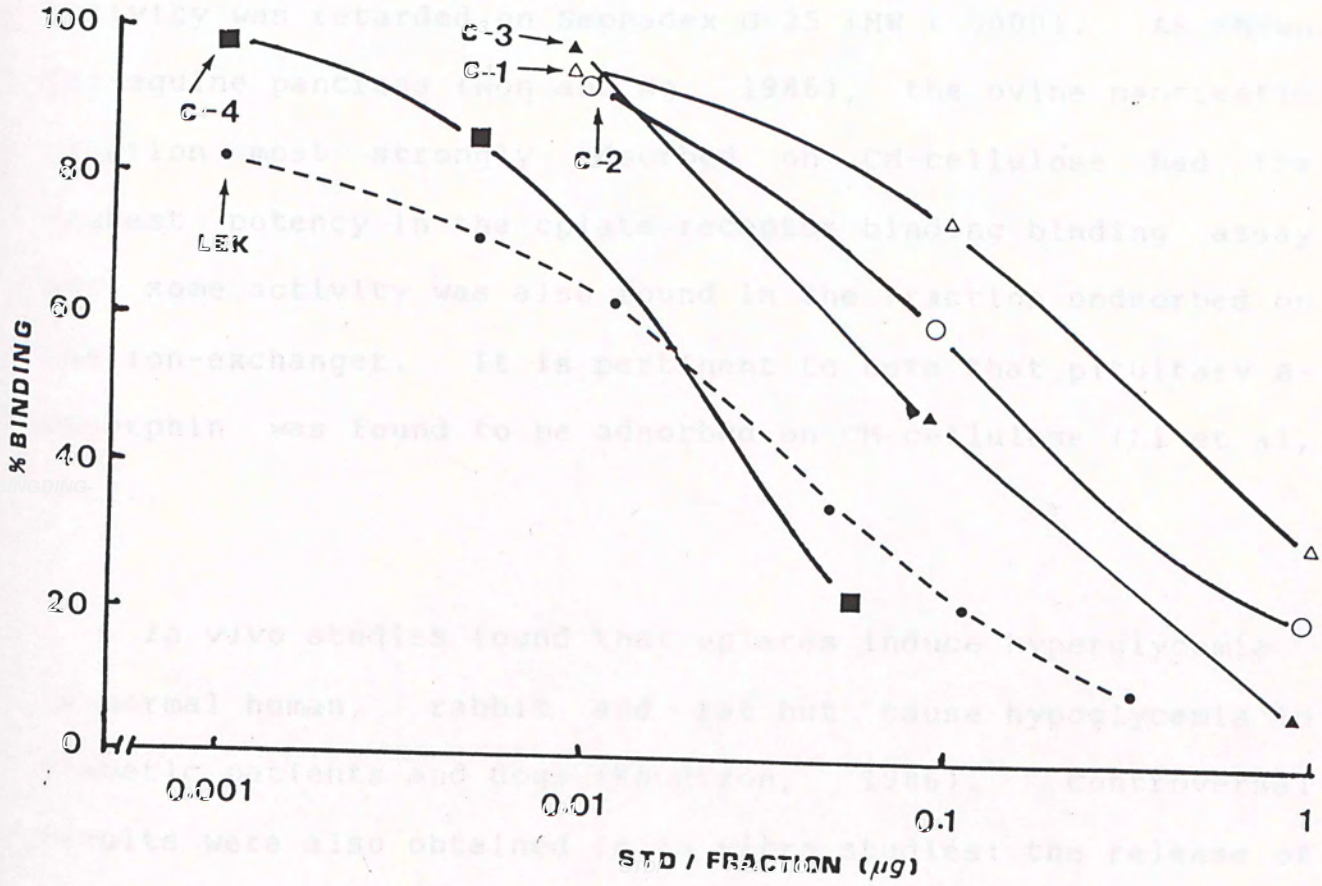
Figure 4-21

Opiate radioreceptor assay data of ovine pancreas fractions from the CM-cellulose column:

Fraction: C1 : OP-1-1-C1;
C2 : OP-1-1-C2;
C3 : OP-1-1-C3;
C4 : OP-1-1-C4.

Standard: LEK (Leucine enkephalin).

Ligand: DADLE.


100
80
60
40
20
0
% BINDING
C-4
C-3
C-1
C-2
LEK
0.001
0.01
0.1
1
STD / FRACTION (μg)

4.3.4 Discussion - ovine pancreas

ß-Endorphin-like substances extracted from the porcine pancreas possessed a molecular weight of approximately 7000 (Houck et al, 1981) while opiate-like substances found in equine pancreas consisted of material with large (MW > 5000) and low (MW < 5000) molecular weight (Hon and Ng, 1986). In the present investigation on ovine pancreas, opiate-like activity was retarded on Sephadex G-25 (MW < 5000). As shown for equine pancreas (Hon and Ng, 1986), the ovine pancreatic fraction most strongly adsorbed on CM-cellulose had the highest potency in the opiate receptor binding binding assay but some activity was also found in the fraction undsorbed on the ion-exchanger. It is pertinent to note that pituitary ß-endorphin was found to be adsorbed on CM-cellulose (Li et al, 1981).

In vivo studies found that opiates induce hyperglycemia in normal human, rabbit and rat but cause hypoglycemia in diabetic patients and dogs (Knudtzon, 1986). Controversal results were also obtained in *in vitro* studies: the release of insulin and glucagon were stimulated secondary to the inhibition of somatostatin release by ß-endorphin in isolated perfused dog pancreas (Ipp et al, 1978) but the result was exactly reverse for insulin and glucagon in isolated perfused rabbit pancreas (Rudman et al, 1983) where somatostatin release remained unchanged. However, pancreastic opiates are believed to affect the pancreatic functions through a paracrine mechanism although the action differs from species

to species. A cell-free translation of the mRNA of a human pancretic tumor yielded POMC and indicated that ACTH and β-lipotropin were produced by the tumor from POMC (Styne et al, 1983).

In the case of pancreatic enkephalin (Stern et al, 1982), it was found that met-enkephalin inhibited the release of pancreatic somatostatin and thus stimulated the release of insulin (Hermansen, 1983). *In vivo* studies also found that the basal release of somatostatin was inhibited by leu-enkephalin (Schusdziarra et al, 1983) However, it is still not known whether it is the local enkephalins or the enkephalins from external neurons that affect the functions of the pancreas.

DATA AND RESULT FROM CLASS AVES

- Pigeon brain

Chapter 5 DATA AND RESULT FROM CLASS AVES - Pigeon brain

5.1 Introduction - ACTH and opiates in birds

Although mammals are conventionally regarded as more highly evolved than birds, the two classes, Mammalia and Aves, actually arose at the same time. However, studies on birds were relatively few compared with those on mammals. POMC, the precursor of ACTH and β-endorphin, together with its mRNA have been found in the pituitary and brain from various mammalian species including the rat, bovine and mouse (Civelli et al, 1982; Jingami et al, 1984) indicating a local synthesizing system of ACTH and β-endorphin in mammalian brains. Localization of preproenkephalin (Yoshikawa et al, 1984; Beaumont et al, 1985) and prodynorphin (Jingami et al, 1984) in mammalian brains has also been reported.

ACTH and β-lipotropin have been found in the turkey pituitary (Chang et al, 1980). Studies on the ostrich pituitary also revealed the presence of ACTH (Li et al, 1978) and β-endorphin (Naude et al, 1981). Since no information on the avian brain was available, a study on the pigeon brain was carried out.

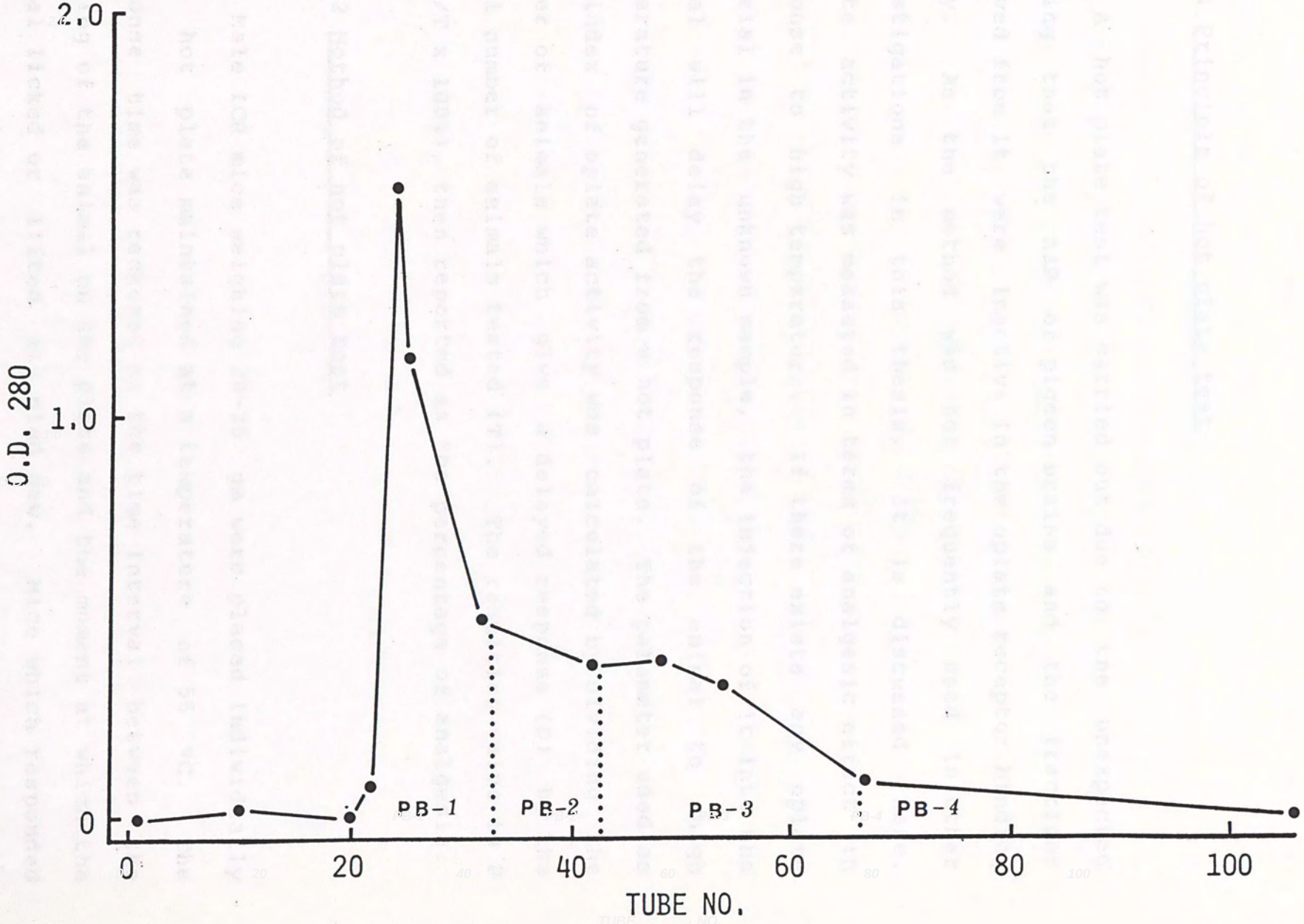
5.2 Extraction of pigeon brain

Pigeon brain acetone powder (AP) was first extracted to yield acid acetone powder (AAP) (section 4.2.2.1). The resulting AAP was dissolved in 0.1 N acetic acid and applied on a Sephadex G-25 column. The resulting column effluent was pooled into four fractions according to their absorbance at 280 nm (figure 5-1). The fractions were then lyophilized and stored at -20 °C until assay for steroidogenic and opiate receptor binding activities and in a hot plate test.

Figure 5-1

Gel filtration of hamster heart AAP (104 mg) on a Sephadex G-25 column (3.5 x 80 cm). Fraction size = 4 ml. Buffer: 0.1 M acetic acid.

Yields: PB-1, 55.6 mg; (Void volume)
PB-2, 11.3 mg;
PB-3, 30.8 mg;
PB-4, trace amount.


2.0
1.0
0
O.D. 280
0
20
40
60
80
100
TUBE NO.
PB-1
PB-2
PB-3
PB-4

5.3 Hot plate test

5.3.1 Principle of hot plate test

A hot plate test was carried out due to the unexpected finding that the AAP of pigeon brains and the fractions derived from it were inactive in the opiate receptor binding assay. As the method was not frequently used in other investigations in this thesis, it is discussed here. Opiate activity was measured in terms of analgesic effect in response to high temperature. If there exists any opiate material in the unknown sample, the injection of it into the animal will delay the response of the animal to high temperature generated from a hot plate. The parameter used as an index of opiate activity was calculated by dividing the number of animals which give a delayed response (D) by the total number of animals tested (T). The resulting value is P = (D/T x 100%), then reported as the percentage of analgesia.

5.3.2 Method of hot plate test

Male ICR mice weighing 20-25 gm were placed individually on a hot plate maintained at a temperature of 55 °C. The response time was reckoned as the time interval between the landing of the animal on the plate and the moment at which the animal licked or lifted its hind paw. Mice which responded between 10 and 20 seconds were selected for the experiment described below. The average response time of this group of

mice and its standard deviation was calculated.

At least 4 hours but no more than 24 hours later, each mouse received an intraperitoneal injection of either saline, morphine or a fraction derived from gel filtration of pigeon brain acid acetone powder on Sephadex G-25. Exactly half an hour later, the animal was placed on the hot plate and the response time was noted. Animals with a response time equal to or greater than the mean response time calculated previously were regarded as a delayed response.

5.4 Results : ACTH-like and opiate-like activities in pigeon brain

From 339 mg pigeon brain acetone powder, 204 mg acid acetone powder was obtained. Table 5-1 presents the result of testing the various G-25 fractions (PB-1, PB-2, PB-3, PB-4) for steroido-genic activity. Only PB-2 stimulated corticosterone production by isolated adrenal decapsular cells. None of the fractions showed activity in the opiate receptor assay when tested up to a concentration of 50 mg/ml. In the hot plate test, morphine at a dose of 0.5 mg/ml (0.1 mg/animal) elicited analgesia in 80% of the assay animals. By contrast, fraction PB-1 at a concentration of 20 mg/ml (4 mg/animal) induced only marginal analgesia. Fractions PB-2 and PB-3 were inactive at the doses tested. A much lower dose had to be employed because of the lower yields of the fractions (table 5-2).

Table 5-1. Steroidogenic activities of pigeon brain fractions eluted from Sephadex G-25

Fraction	Dose (mg)	Steroidogenic activity (ng corticosterone/hr/25,000 cells)
Control	0	UD
ACTH	1 (nM)	0.74 ± 0.25
"	0.3 (nM)	0.35 ± 0.03 ^
"	0.1 (nM)	0.04 ± 0.001 ^
"	0.012 (nM)	0.029 ± 0.006
PB-1	1	UD
"	0.1	UD
"	0.01	UD
"	0.001	UD
PB-2	0.05	0.061 ± 0.001 ^
"	0.005	0.023 ± 0.003 ^^
PB-3	1	UD
"	0.1	UD
"	0.01	UD
PB-4	1	UD
"	0.1	UD

Values are represented as mean ± S.E.M. of triplicate determinations.

UD : Undetectable
^ : $p < 0.001$ compared with control
^^ : $p < 0.005$ compared with control

Table 5-2. Hot plate test of the pigeon brain fractions eluted from Sephadex G-25 for analgesic activity

Fraction	Dose (mg/ml)	Response time of individual mouse (sec)	Analgesia (%)
Saline	0	12 (-) 13 (-) 11 (-) 12 (-)	0
Morphine	0.5	25 (+) 23 (+) 60 (+) 25 (+) 21 (-)	80
PB-1	20	15 (-) 19 (-) 12 (-) 24 (+) 20 (-)	20
PB-2	0.8	15 (-) 13 (-) 14 (-) 9 (-) 17 (-)	0
PB-3	16.7	15 (-) 16 (-) 14 (-) 15 (-) 13 (-)	0
PB-4	0.2	8 (-) 7 (-) 12 (-) 12 (-) 4 (-)	0

Mean response time + 3 SD = 15.03 + 8.27 = 23.3 (sec)

(+) : Analgesic effect, response time ≥ 23 sec.
(-) : No analgesic effect, response time smaller than 23 sec.

The volume of samples injected was 200 µl/mouse

5.5 Discussion - pigeon brain

The presence of ACTH-like material with molecular weight less than 5000 was demonstrated in the pigeon brain. The failure of the fractions derived from pigeon brain AAP to displace DADLE in the opiate receptor binding assay or elicit a delayed response in the hot plate test could be due to the absence or the presence of only trace amounts of opiate-like material in the pigeon brain, especially that specifically bind to δ- and/or μ-receptors. Other possibilities that cannot be ruled out include the presence of N-acetylated derivatives which have no receptor binding or biological activity (Li et al, 1980), the presence of molecules with substitutions in the sequence by amino acids that result in little or no activity in the mammalian recipient, and loss during purification.

ACTH was found to stimulate the secretion of both aldosterone (Radke et al, 1984) and corticosterone (Klingbeil, 1985) in birds. Findings from ducks suggest that ACTH produces similar secretory patterns from the two zones of the adrenal cortex in birds compared with mammals (Hauing, 1970; Klingbeil, 1985), indicating that the role of ACTH in birds is very similar to that in mammalian species. Furthermore, studies in the pigeon revealed a relation of ACTH to exercise (Rees and Harvey, 1987) as in mammals. ACTH exerts apparent effects in cognitive functions such as learning and memory in mammals (Krieger, 1984). Whether the ACTH-like material found

here in pigeon brain has similar functions remains to be elucidated. Moreover, as the pars distalis lies apart from the brain in birds (Kobayashi and Wada, 1973), it is not too likely that the ACTH-like material found in the pigeon brain originated from the pituitary gland.

DATA AND RESULT FROM CLASS REPTILIA

- Snake and turtle

Chapter 6 DATA AND RESULT FROM CLASS REPTILIA - Snake and turtle

6.1 Introduction - ACTH and opiates in reptiles

The studies on the so-called warm-blooded vertebrates - mammals and birds, were described in the last chapter. The results obtained from the tissues of lower vertebrates will be discussed in this and the subsequent chapters. Among these vertebrates, the one that lives on land, the reptile, is discussed in this chapter. As reptiles do not maintain a constant temperature, they cannot maintain a highly efficient nervous system like mammals and also cannot tolerate long-term vigorous exercise like birds. The standard metabolic rate of reptile is only 10-20 per cent of that in homeotherms (Bennet and Dawson, 1976).

Although the phylogenetic order is followed in this thesis, the case is not also true when considering the amount of researches in the literature. Studies on ACTH and opiates in reptile are actually more extensive compared with those on the birds. POMC-derived peptides have been found in the lizard (*Anolis carolinensis*) pituitary (Dores and Surprenant, 1984) and brain (Dores et al, 1984a) by histochemical methods. β-Endorphin, the opioid peptide derived from POMC, was found in the brain of another lizard (*Lacerta muralis*) by an immunocytochemical method (Vallarino, 1986). Recently, proenkephalin was also reported in the brains of all the three

reptilian subclasses (Lindberg and White, 1986) represented by lizard, alligator, and turtle. Then the presence of POMC-related peptides and opiates in the brain and pituitary seems to be a universal event in reptiles. However, this hypothesis can only be proved to be right or wrong by research done on other different reptiles.

In this chapter, The species chosen for study were the turtle and snake which belong to two different subclasses in Class Reptilia. The pituitary gland and brain of the snake will be discussed first and then followed by the heart and intestine of turtles.

6.2 Snake brain

6.2.1 Introduction - ACTH and opiates in snake brain

In comparison to mammals and birds, the reptilian brain is relatively small, being, at most, 1 per cent of the body weight (Young, 1983b) and less differentiated (Webster and Webster, 1974).

Snakes are extremely specialized legless reptiles and they belong to a different suborder from lizards. Compared with the lizard, the internal organs of the snake are completely rearranged to fit to its long and narrow body cavity (Pough, 1979). Its highly specialized methods of locomotion and predation reflect a specialized coordination system in the body. So, there is no reason to think that the case in the lizard (section 6.1) is also applicable to the snake especially with regard to the brain which coordinates all the physiological functions of the body. Among the various snakes, the presence of POMC-derived peptides was only reported in the brain of the sea snakes, *Lapemis hardwickii* and *Hydrophis cyanocinctuswas*, recently (Ng et al, 1986). However, only the crude acid acetone powder was used in their study and the nature of these hormone-like activities in the snake brain had not yet been characterized. Hence the presence of POMC or other opiate precursors in the snake brain remains a question. In view of this, the present study on the freshwater snake, *Ptyas mucosa*, was undertaken.

6.2.2 Materials and methods - extraction of snake brain

The protocol for the extraction of snake brains is shown in figure 6-1. First, acid acetone powder (AAP) was prepared from snake brains. The AAP was then loaded on a Sephadex G-25 column and the resulting fractions were further purified by ion-exchange chromagraphy on a CM-cellulose column.

6.2.2.1 Preparation of snake brain acid acetone powder (AAP)

The AAP of snake brain was prepared with the method used for the preparation of rat heart AAP (section 4.1.2.1a).

6.2.2.2 Chromatographic purification of snake brain AAP

The snake brain acid acetone powder (AAP) was first dissolved in 0.1 N acetic acid and then subjected to gel filtration on Sephadex G-25 and eluted with 0.1 N acetic acid. The effluent was divided into three separate fractions according to absorbance at 280 nm (Figure 6-2). The first fraction eluted at the void volume and so apparently has a molecular weight of over 5000. The first two high molecular weight fractions, which were obtained with a higher yield and possessed higher biological activities, were then lyophilized and re-dissolved in 10 mM ammonium acetate (pH = 4.6) and subjected to ion-exchange chromatography on a carboxymethyl (CM)-cellulose column. After the unadsorbed material had been eluted, the column was further eluted with combined ionic (10 mM - 0.5 M) and pH (4.6 - 7) gradients established with

Figure 6-1 Extraction and purification of ACTH-like and opiate-like activities from snake brain

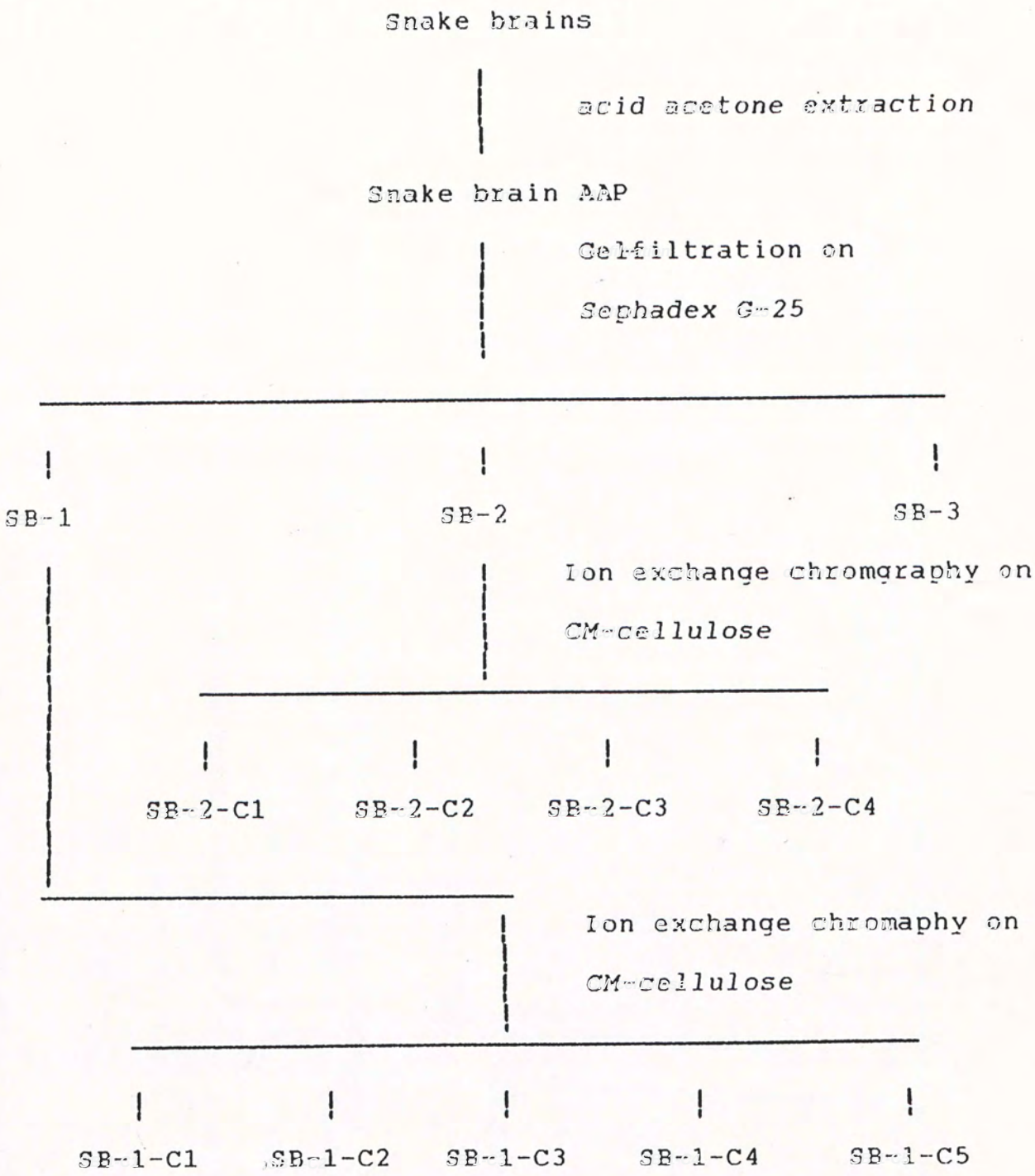
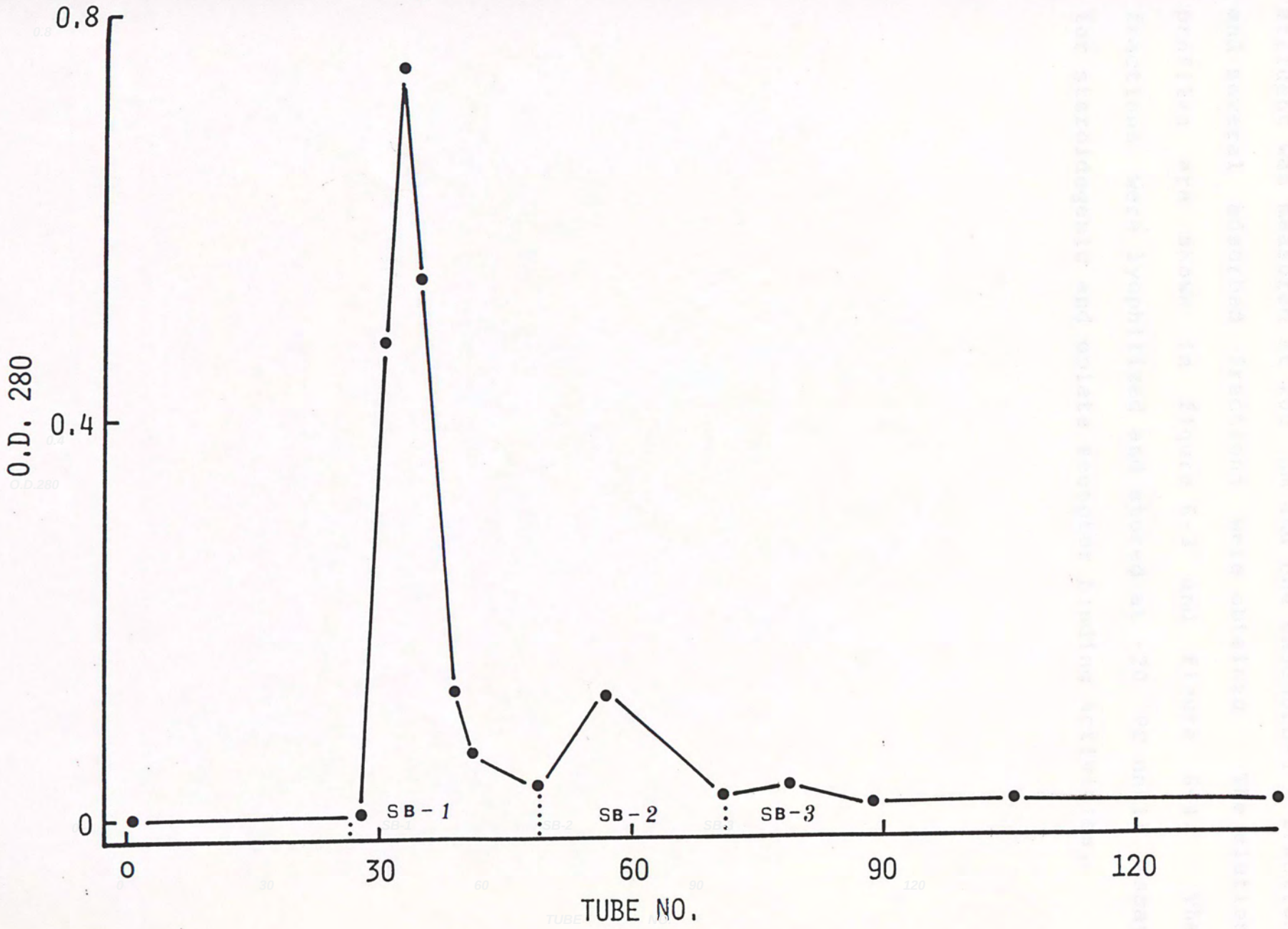


Figure 5-2

Gel filtration of snake brain AAP (160 mg) on a Sephadex G-25 column (3.5 x 80 cm). Fraction size = 5 ml.

Buffer: 0.1 M acetic acid.

Yields: SB-1, 78 mg; (Void volume)
SB-2, 59 mg;
SB-3, 3.3 mg.


0.8
0.4
0
O.D. 280
SB-1
SB-2
SB-3
0
30
60
90
120
TUBE NO.

ammonium acetate in order to recover the adsorbed materials based on their ionic properties. Again, the absorbance of the effluent was measured at 280 nm and one unadsorbed fraction and several adsorbed fractions were obtained The elution profiles are shown in figure 6-3 and figure 6-4. The fractions were lyophilized and stored at -20 °C until assay for steroidogenic and opiate receptor binding activities.

Figure 6-3

Ion exchange chromatography of snake brain fraction SB-1 (302.5 mg) on a CM-cellulose column (1.4 x 36 cm). Fraction size = 5 ml.

Buffer: 10 mM NH_4OAc, pH 4.6 (fractions 1-20);
10 - 100 mM NH_4OAc, pH 4.6 - 6.7 (fractions 21-60);
0.1 - 0.2 M NH_4OAc, pH 6.7 - 7 (fractions 61-100);
0.2 - 0.5 M NH_4OAc, pH 7 (fractions 100-140);
0.5 M NH_4OAc, pH 7 (fractions 141-160).
12.5 M NH_4OH, pH 11; (column eluted with one bed volume of this buffer, profile not recorded)

Yields: SB-1-C1, 9.6 mg;
SB-1-C2, 91.6 mg;
SB-1-C3, 35.5 mg;
SB-1-C4, 82.7 mg;
SB-1-C5, 64 mg. (pool of fractions eluted by NH OH)

(1) : pH gradient
(2) : ionic gradient
(M) : Molarity of eluent

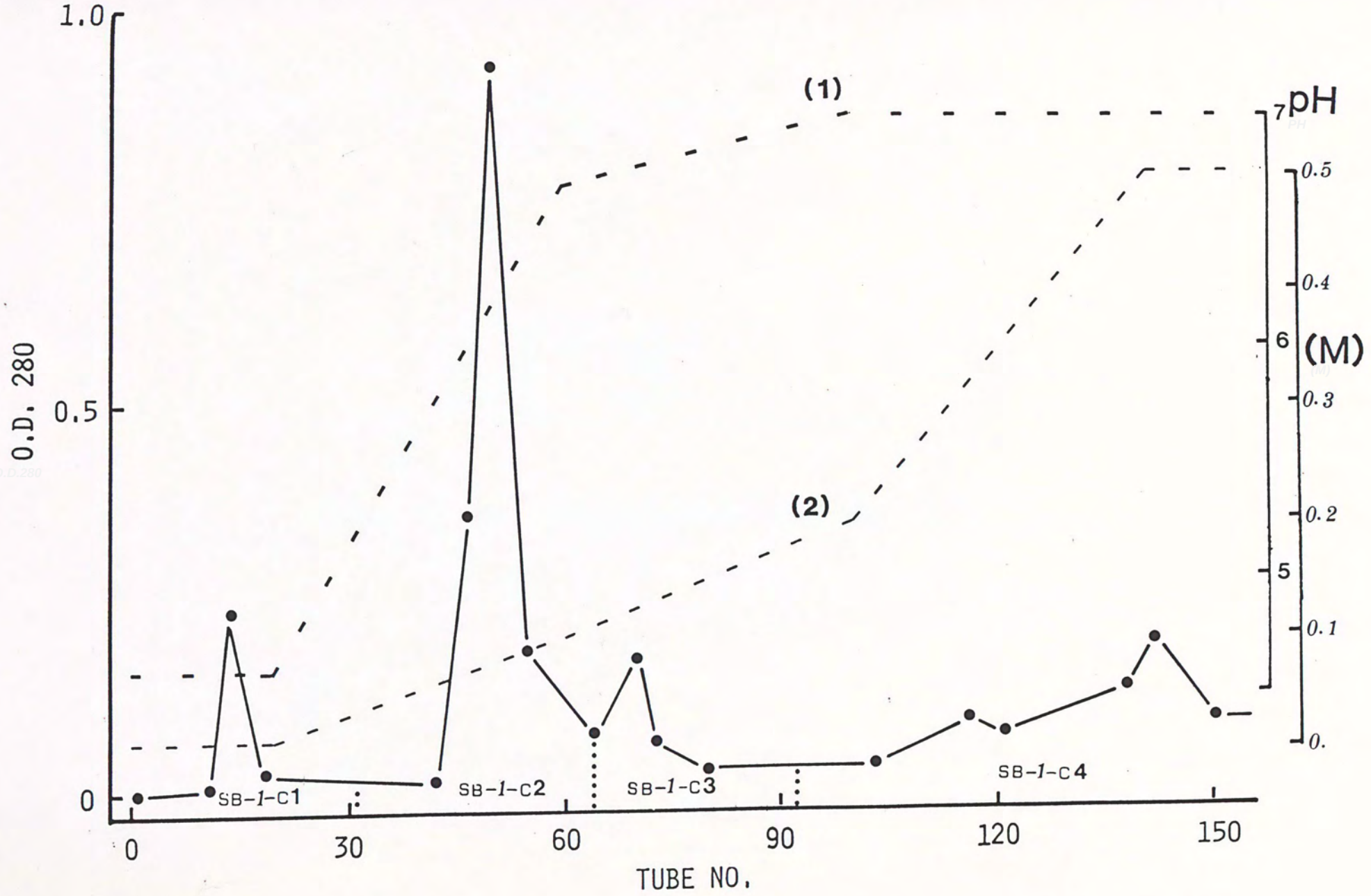

1.0
0.5
0
O.D. 280
(1)
(2)
pH
7
6
5
(M)
0.5
0.4
0.3
0.2
0.1
0.
SB-1-C1
SB-1-C2
SB-1-C3
SB-1-C4
0
30
60
90
120
150
TUBE NO.

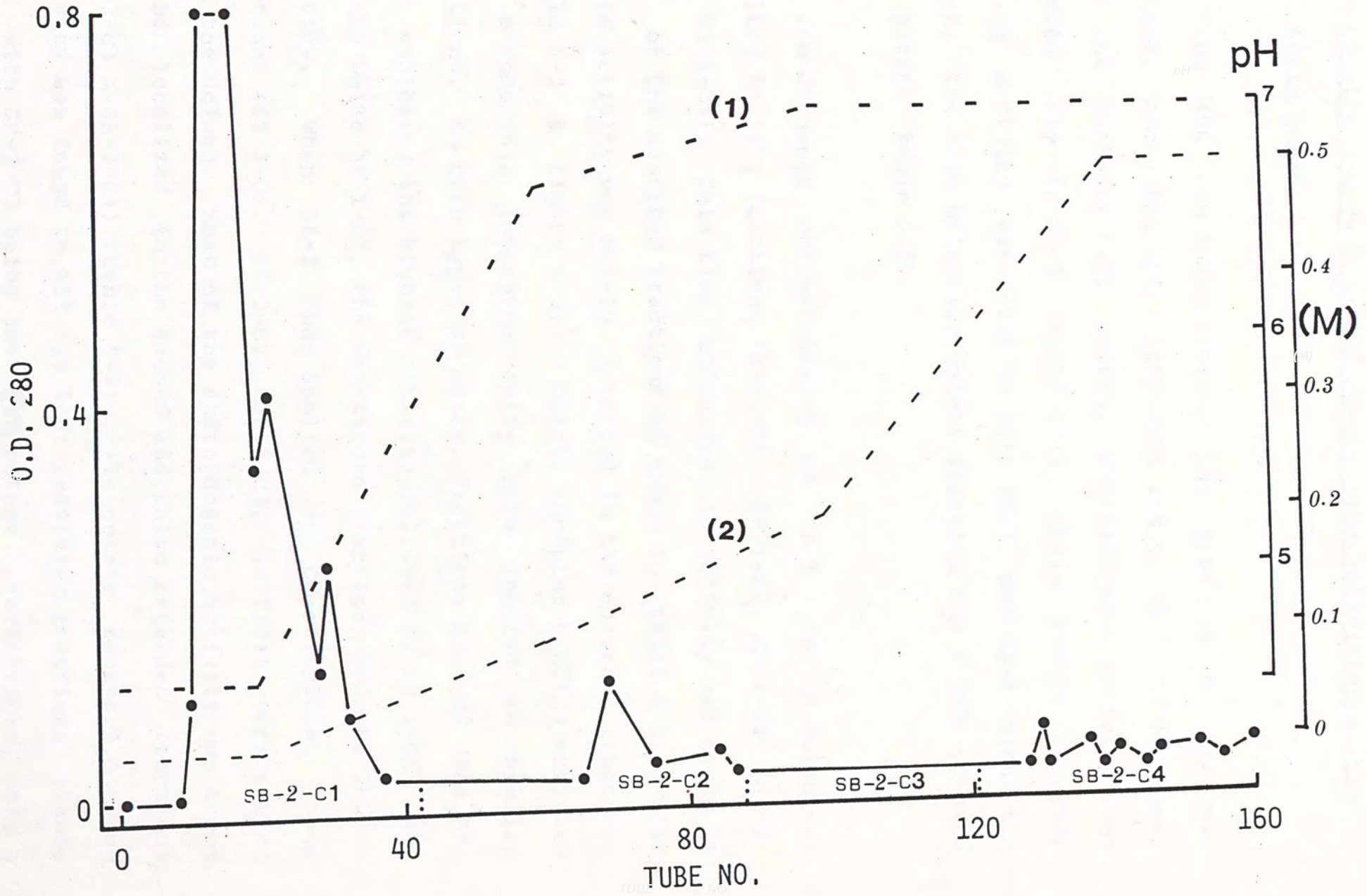
Figure 6-4

Ion exchange chromatography of snake brain fraction SB-1 (500 mg) on a CM-cellulose column (1.4 x 36 cm). Fraction size = 5 ml.

Buffer: 10 mM NH_4OAc, pH 4.6 (fractions 1-20);
10 - 100 mM NH_4OAc, pH 4.6 - 6.7 (fractions 21-60);
0.1 - 0.2 M NH_4OAc, pH 6.7 - 7 (fractions 61-100);
0.2 - 0.5 M NH_4OAc, pH 7 (fractions 101-140);
0.5 M NH OAc, pH 7 (fractions 141 - 160).

Yields: SB-2-C1, 380 mg;
SB-2-C2, 11 mg;
SB-2-C3, 20 mg;
SB-2-C4, 24 mg.

(1) : pH gradient
(2) : ionic gradient
(M) : Molarity of eluent


0.8
0.4
0
O.D. 280
pH
7
6
5
(M)
0.5
0.4
0.3
0.2
0.1
0
(1)
(2)
SB-2-C1
SB-2-C2
SB-2-C3
SB-2-C4
0
40
80
120
160
TUBE NO.

6.2.3 Results : ACTH-like and opiate-like activities in the snake brain

From 400 gm snake brains, only 5.64 gm of AAP was obtained. Among the three fractions (SB-1, SB-2, SB-3) eluted from the Sephadex G-25 column, steroidogenic activity was observed only in SB-2 (table 6-1) while opiate receptor binding activity was found in both SB-1 and SB-2 with the former, the high molecular weight fraction (MW > 5000), being more potent (table 6-1).

Ion-exchange chromatography of SB-1 on CM-cellulose resulted in five fractions (SB-1-C1, SB-1-C2, SB-1-C3, SB-1-C4, SB-1-C5). This time, steroidogenic activity was found in some of the adsorbed fractions as shown in table 6-2 while opiate activity was mainly detected in the adsorbed fractions (table 6-2 & figure 6-5). Opiate receptor binding activity and β-endorphin immunoreactivity were located in similar fractions. In both types of assay, fractions SB-1-C3 and SB-1-C4 exhibited the highest activity followed by SB-1-C5 and SB-1-C2 while SB-1-C1, the unretarded fraction, had the lowest activity. When SB-2 was applied on CM-cellulose, four fractions (SB-2-C1, SB-2-C2, SB-2-C3, SB-2-C4) were eluted from the column. Most of the steroidogenic activity was found to be localized in the second and third retarded fractions (SB-2-C3 & SB-2-C4) (table 6-3) while opiate receptor binding activity was found in all the four resulting fractions (table 6-3) with SB-2-C3 being the most potent. Furthermore, only a very small portion of the ACTH- and opiate-like activities

Table 5-1. Steroidogenic and opiate activities of snake brain fractions eluted from Sephadex G-25

Fraction	Steroidogenic activity		Opiate receptor binding activity	
	Dose (mg)	(ng corticosterone /hr/25,000 cells)	Dose (mg)	%Binding
Control	0	0.090 ± 0.013	-	-
ACTH	1 (nM)	1.603 ± 0.072 ^	-	-
"	0.2 (nM)	0.580 ± 0.048 ^	-	-
"	0.04 (nM)	0.140 ± 0.002	-	-
"	0.008 (nM)	0.086 ± 0.005	-	-
LEK	-	-	4 (µg)	16.8 ± 1.34 ^
"	-	-	0.8 (µg)	38.1 ± 3.10 ^
"	-	-	0.16 (µg)	79.0 ± 2.76 ^
"	-	-	0.032 (µg)	104.9 ± 2.99
SB-1	1	UD	0.5	5.8 ± 0.51
"	0.1	0.008 ± 0.002	0.05	41.0 ± 3.68
SB-2	0.1	0.114 ± 0.004	0.5	52.2 ± 2.23
"	0.01	0.023 ± 0.008	0.05	116.9 ± 2.07
SB-3	1	UD		UD

Values represent mean ± S.E.M. of triplicate determinations.

LEK : Leu-enkephalin
UD : Undetectable
^ : $p < 0.001$ compared with control or buffer
^^ : $p < 0.005$ compared with control or buffer

Table 6-2. Steroidogenic and opiate activities of CM-cellulose fractions derived from snake brain fraction: SB-1

Fraction	Steroidogenic activity		Opiate receptor binding activity	
	Dose (mg)	(ng corticosterone /hr/25,000 cells)	Dose (mg)	%Binding
Control	0	0.011 ± 0.003	-	-
ACTH	2 (nM)	0.654 ± 0.085 ^^	-	-
"	0.2 (nM)	0.003 ± 0.001	-	-
LEK	-	-	0.37 (µg)	25.7 ± 7.26 ^
"	-	-	0.123 (µg)	48.9 ± 3.11 ^
"	-	-	0.041 (µg)	70.3 ± 3.54 ^^
"	-	-	0.002 (µg)	102.4 ± 0.25
SB-1	-	-	0.5	53.8 ± 5.59 ^^
"	-	-	0.05	94.0 ± 3.54
"	-	-	0.005	101.9 ± 2.21
SB-1-C1	1	0.009 ± 0.001	0.5	86.4 ± 4.17
"	0.1	0.006 ± 0.002	0.05	106.3 ± 2.60
"	0.01	0.002 ± 0.000	0.005	105.2 ± 3.97
SB-1-C2	1	0.022 ± 0.001	0.5	60.2 ± 1.50 ^
"	0.1	0.076 ± 0.010 ^^	0.05	97.1 ± 2.47
"	0.01	0.020 ± 0.000	0.005	94.9 ± 0.12
SB-1-C3	1	UD	0.5 (β+)	32.1 ± 1.88 ^
"	0.1	0.273 ± 0.216	0.05	82.7 ± 4.86
"	0.01	0.758 ± 0.620	0.005	123.0 ± 0.74
SB-1-C4	1	UD	0.5	31.6 ± 2.24 ^
"	0.1	0.015 ± 0.001	0.05	73.2 ± 2.79 ^^
"	0.01	0.175 ± 0.012 ^	0.005	94.9 ± 3.13
SB-1-C5	1	0.050 ± 0.006 ^^	0.5	70.1 ± 6.82
"	0.1	0.556 ± 0.153	0.05	115.0 ± 5.25
"	0.01	0.951 ± 0.086 ^	0.005	110.7 ± 2.21

Values represent mean ± S.E.M. of triplicate determinations.

LEK : Leu-enkephalin
UD : Undetectable
^ : $p < 0.001$ compared with control or buffer
^^ : $p < 0.005$ compared with control or buffer
(β+) : Most potent fraction in β-endorphin RIA

Figure 6-5

Displacement of [^{125}I]β-endorphin from β-endorphin antiserum by the fractions of CM-cellulose derived from the first fraction (SB-1) of Sephadex G-25 of snake brain

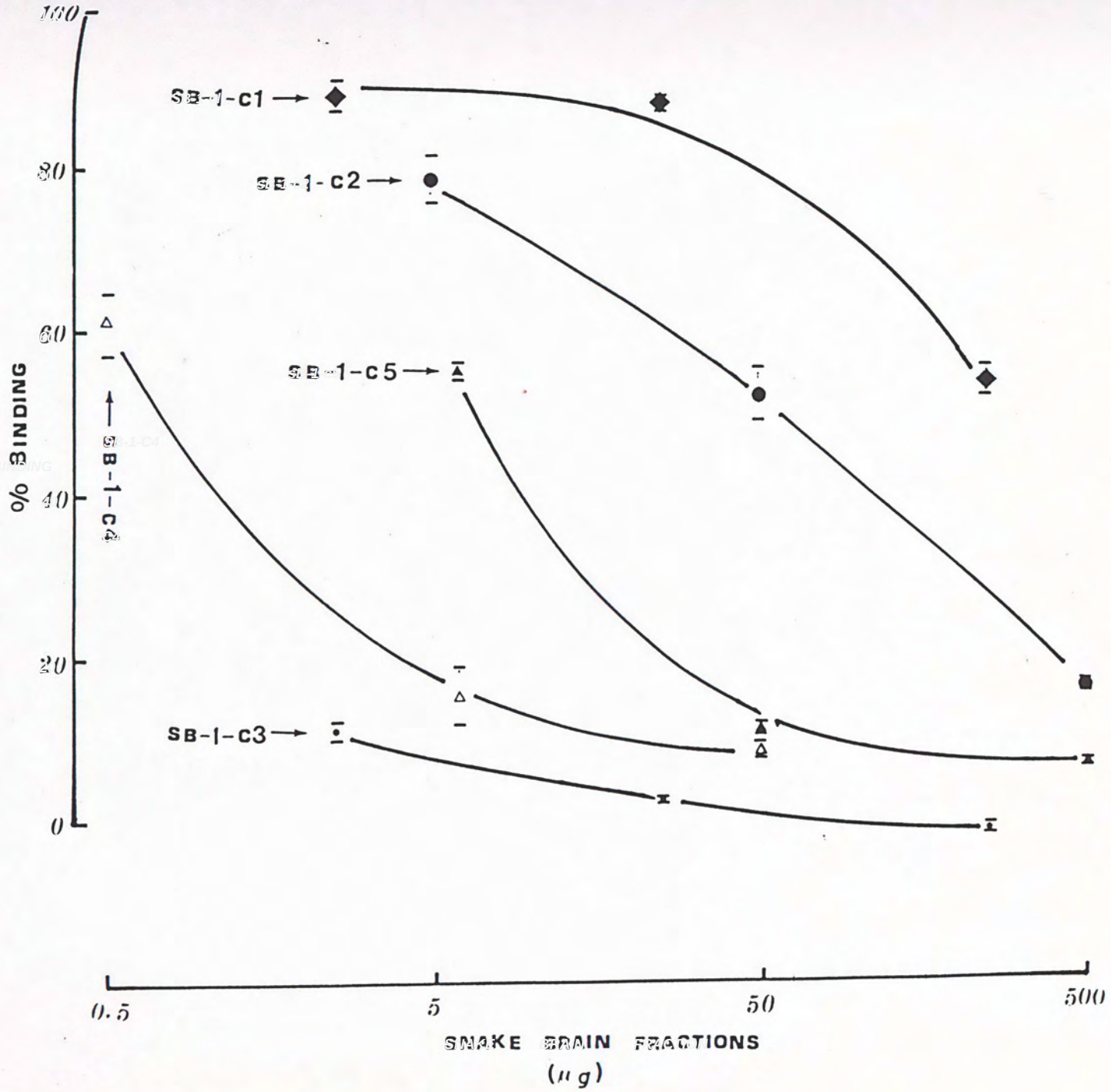

100
80
60
40
20
0
% BINDING
SB-1-c1
SB-1-c2
SB-1-c5
SB-1-c4
SB-1-c3
0.5
5
50
500
SNAKE BRAIN FRACTIONS
(μg)

Table 6-3. Steroidogenic and opiate activities of CM-cellulose derived from snake brain fraction: SB-2

Fraction	Steroidogenic activity		Opiate receptor binding activity	
	Dose (mg)	(ng corticosterone /hr/25,000 cells)	Dose (mg)	%Binding
Control	0	0.090 ± 0.013	-	-
ACTH	1 (nM)	1.511 ± 0.082 ^	-	-
"	0.33 (nM)	1.154 ± 0.065 ^	-	-
"	0.11 (nM)	0.551 ± 0.036 ^	-	-
"	0.037 (nM)	0.205 ± 0.005 ^^	-	-
"	0.012 (nM)	0.080 ± 0.027	-	-
"	0.004 (nM)	0.053 ± 0.003	-	-
LEK	-	-	4 (µg)	16.8 ± 1.34 ^
"	-	-	0.8 (µg)	38.1 ± 3.10 ^
"	-	-	0.16 (µg)	79.0 ± 2.76 ^
"	-	-	0.032 (µg)	104.9 ± 2.99
SB-2-C1	4	0.001 ± 0.000	0.5	63.1 ± 3.26 ^
"	0.4	0.099 ± 0.046	0.05	84.8 ± 9.20
SB-2-C2	1	0.055 ± 0.013	0.5	77.9 ± 11.36
"	0.1	0.059 ± 0.011	0.05	116.9 ± 2.07
SB-2-C3	0.5	0.608 ± 0.016 ^	0.5	5.8 ± 0.51 ^
"	0.125	0.569 ± 0.027 ^	0.05	41.0 ± 3.68 ^
"	0.031	0.105 ± 0.015		
SB-2-C4	0.1	0.429 ± 0.034 ^	0.5	29.2 ± 2.96 ^
"	0.01	0.023 ± 0.008	0.05	92.0 ± 4.55

Values represent mean ± S.E.M. of triplicate determinations.

LEK : Leu-enkephalin
^ : $p < 0.001$ compared with control or buffer
^^ : $p < 0.005$ compared with control or buffer

were found in the unadsorbed fractions from the CM-cellulose column as in previous studies on mammalian species.

6.2.4 Discussion - snake brain

The results suggest the presence of ACTH-like and opiate-like activities of various molecular weight and different chromatographic behaviour on CM-cellulose in the snake brain. ACTH-like material was also found in the pigeon brain (Chapter 5). It is likely that ACTH is an ancient molecule and present in the brains of lower vertebrates other than the homeotherms. High molecular weight ACTH-like and opiate-like materials (table 6-2) were also found in the snake brain and co-eluted from the CM-cellulose column (SB-1-C-4). β-Endorphin-like immunoreactivity was also found in the high molecular fraction (SB-1) derived from Sephadex G25. As β-endorphin and ACTH were derived from a common precursor, POMC, in mammals, whether these finding indicate the presence of any possible precursor molecule remains to be studied. On the other hand, the nature of the opiate-like activities of the CM-cellulose fractions derived from low molecular weight SB-2 fraction have not been studied due to insufficient material left for the β-endorphin RIA assay.

6.3 Snake pituitary

6.3.1 Introduction - ACTH and opiates in the snake pituitary

The studies on the presence of ACTH and opiates in the snake pituitary are even fewer in number than that on the snake brain if there is any. The structure of the reptilian pituitary, compared with those of mammals and avians, has a more enlarged pars intermedia which secretes MSH, a peptide derived from POMC and serves as a colour producer in the body (Holmes and Ball, 1974a).

6.3.2 Materials and methods - extraction of snake pituitaries

The protocol is shown in figure 6-6. Two methods have been applied in preparing snake pituitary to compare their effectiveness in purification. Method I was similar to the methods of purifications used throughout this thesis while method II used a completely different one.

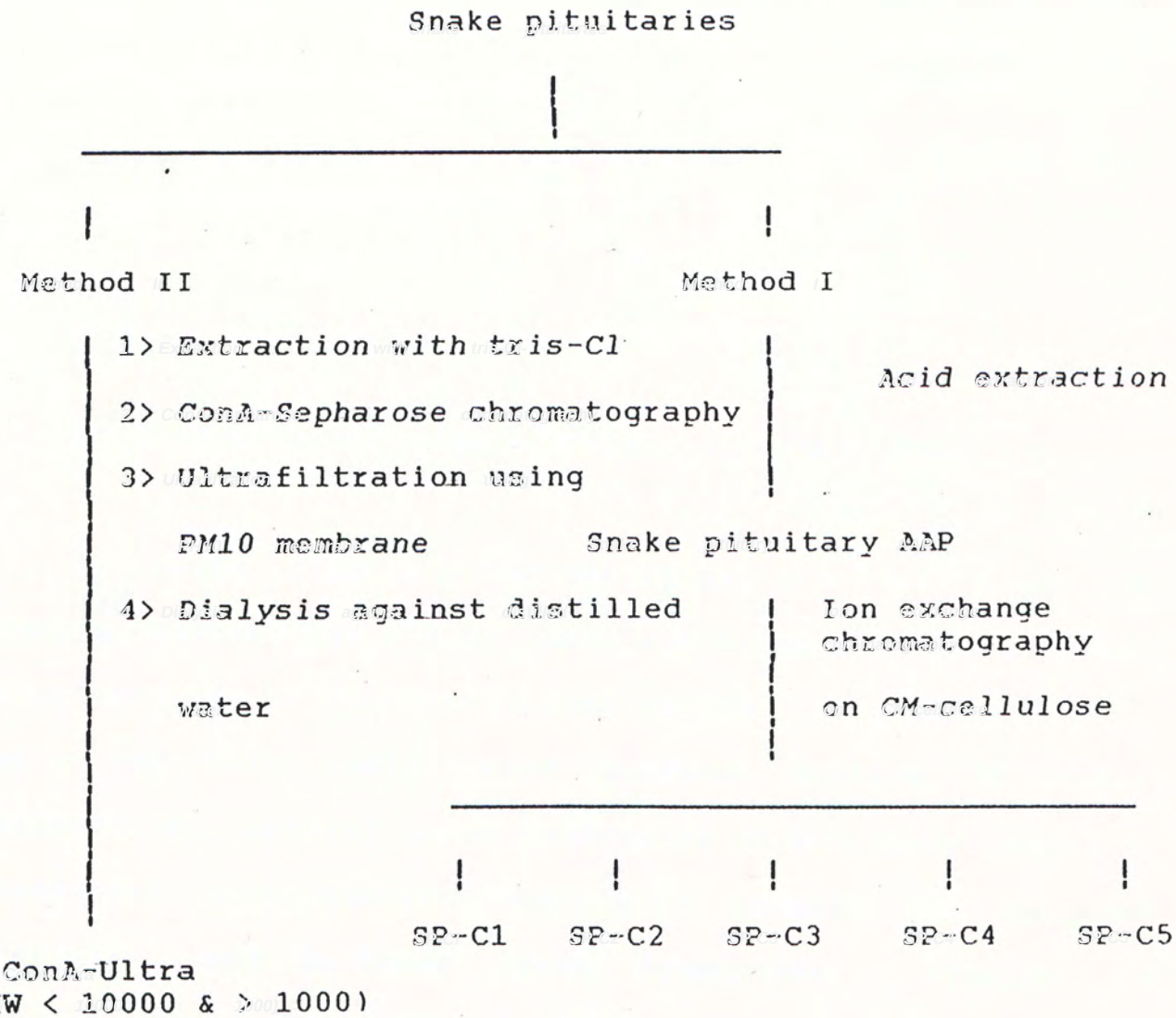
6.3.2.1 Method I

Unlike the case of snake brains, the starting amount of snake pituitaries available was very small. Assuming the same percentage yield, It has been calculated that the resulting AAP of snake pituitary would have been only about 10 mg if the usual AAP preparation method (section 4.1.2.1) had been applied. Thus, an acid extraction method was applied here instead of a complete acid acetone extraction procedure. However, for con-venience, the resulting acid extract was also called AAP. The AAP was then applied to the CM-cellulose column for ion-exchange chromatographic purification (figure 6-6).

6.3.2.1a Preparation of snake pituitary AAP using acid extraction method

The snake pituitaries were first rinsed in 0.9% saline at 4 °C to remove blood. The tissues were then suspended in 5 ml of an acidic medium (1 N acetic acid & 0.1 N HCl). The mixture was heated at 90 °C for 15 minutes. It was then

Figure 6-6 Extraction and purification of ACTH-like and opiate-like activities from snake pituitary



cooled in ice and homogenized in a blender at 4 °C. The blender was rinsed with another 5 ml of the same medium to minimize any loss of sample. The combined (10 ml) mixture was then centrifuged at 12,000 g at 4 °C for 15 minutes. The resulting pellet was discarded and the supernatant was lyophilized. The material (AAP) was re-lyophilized with 1 to 2 ml distilled water before storage in -20 °C until further processing.

6.3.2.1b Chromatographic purification of snake pituitary AAP

The snake pituitary AAP was first dissolved in 10 mM ammonium acetate (pH = 4.6) and subjected to ion-exchange chromatography on a CM-cellulose column. As the amount of sample was very small, step gradient instead of linear gradient was applied using increasing pH and ionic strength established with ammonium acetate to recover the adsorbed materials based on their ionic properties. Finally, a solution of ammonium hydroxide (0.6 M, pH 10.6) was used to elute any materials strongly adsorbed on the CM-cellulose column. The absorbance of the effluent was measured at 280 nm. The elution profile of snake pituitary AAP on CM-cellulose is shown in figure 6-7.

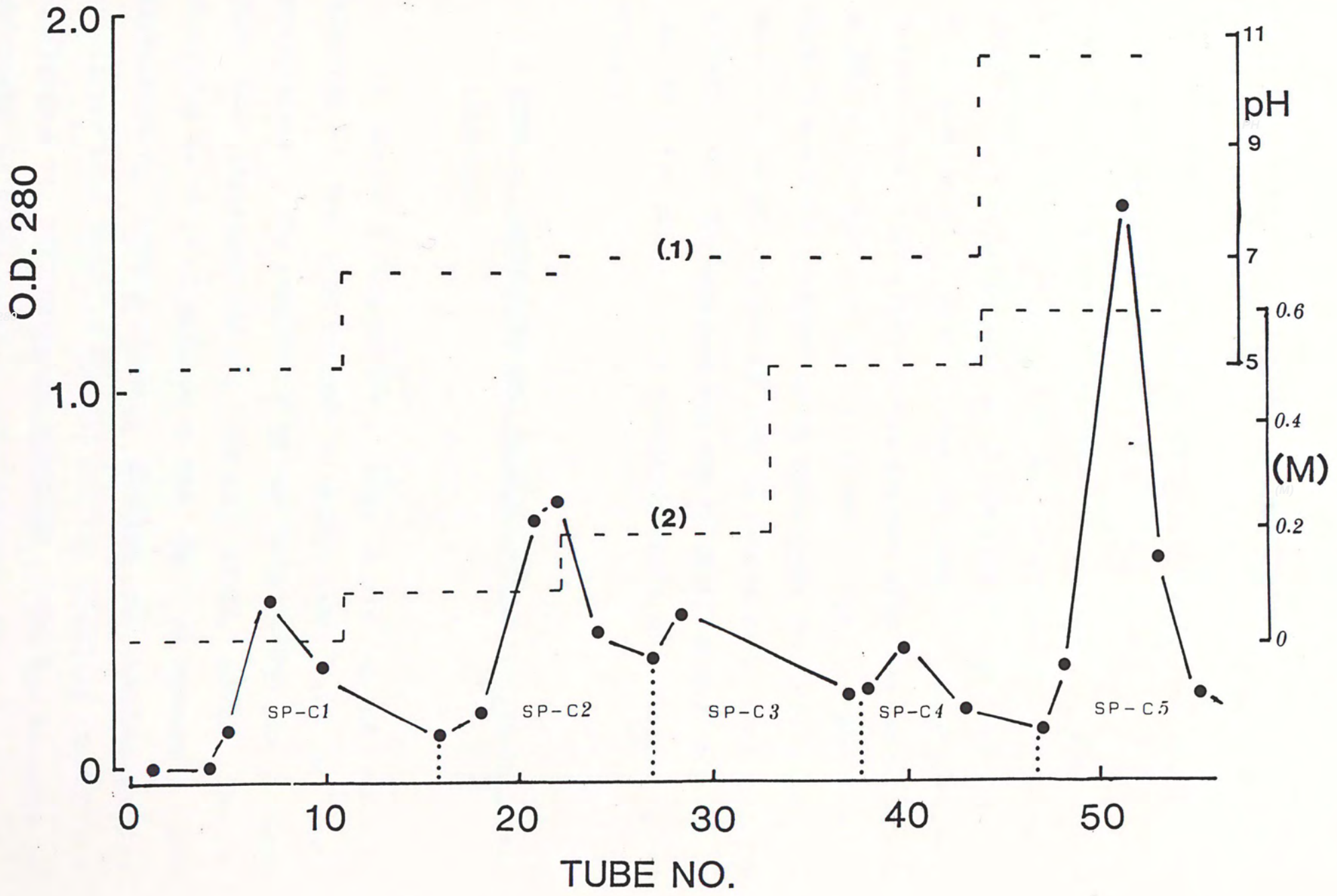
Figure 6-7 Snake pituitary AAP on CM-cellulose

Ion exchange chromatography of snake pituitary AAP on a CM-cellulose column (1.2 x 17 cm). The AAP (71 mg) was dissolved in starting buffer, centriguged and the resulting supernatant applied on the column. Fraction size = 3 ml.

Buffer: 10 mM NH_4OAc, pH 4.6 (fractions 1-11);
100 mM NH_4OAc, pH 6.7 (fractions 12-22);
0.2 M NH_4OAc, pH 7 (fractions 23-33);
0.5 M NH_4OAc, pH 7 (fractions 33-44);
0.6 M NH_4OH, pH 10.6; (fractions 45-55).

Yields: SP-C1, 2 mg;
SP-C2, 11.3 mg;
SP-C3, 5 mg;
SP-C4, 1.4 mg;
SP-C5, 8.5 mg.

(1) : pH gradient
(2) : ionic gradient
(M) : Molarity of eluent


2.0
1.0
0
O.D. 280
(1)
(2)
11
9
7
5
pH
0.6
0.4
0.2
0
(M)
SP-C1
SP-C2
SP-C3
SP-C4
SP-C5
0
10
20
30
40
50
TUBE NO.

6.3.2.2 Method II

As this method was seldom applied in this thesis, it will not be discussed in detail. The protocol is shown in figure 6-6. Snake (*Ptyas mucosa*) pituitaries were homogenized in tris-Cl buffer at pH 7.8. The extract was then chromatographed on ConA-Sepharose which adsorbs glycoproteins such as TSH and gonadotropins. The unadsorbed nonglycoprotein fraction was then concentrated through PM10 membrane with a molecular weight cut-off of 10,000. The filtrate (MW < 10,000) was then dialyzed using Spectropor dialysis bag with a molecular weight cut-off of 1000 to remove salt. Finally the content of the dialysis bag was lyophilized and stored at -20 °C until assay. The resulting fraction is called 'ConA-Ultra'.

6.3.3 Results : ACTH-like and opiate-like activities in snake pituitary

In method I (figure 6-6), only 0.074 gm of AAP was obtained by the acid extraction method from 0.9 gm snake pituitaries. The effluent from the CM-cellulose was divided into five fractions (SP-C1, SP-C2, SP-C3, SP-C4, SP-C5) according to their absorbance at 280 nm. As expected, both steroidogenic, opiate receptor binding and β-endorphin-like activities were found in the AAP and the fractions (table 6-4 & figure 6-8) indicating the presence of the two hormones in pituitary of *Ptysa mucosa*. Furthermore, the activities of ACTH obtained was the highest compared with any other

Table 6-4. Steroidogenic and opiate activities of snake pituitary fractions eluted from CM-cellulose

Fraction	Steroidogenic activity		Opiate receptor binding activity	
	Dose (mg)	(ng corticosterone /hr/25,000 cells)	Dose (mg)	%Binding
Control	0	0.036 ± 0.007	-	-
ACTH	2 (nM)	0.351 ± 0.053 ^^	-	-
"	0.5 (nM)	0.096 ± 0.020	-	-
"	0.125 (nM)	0.052 ± 0.009	-	-
"	0.031 (nM)	0.028 ± 0.002	-	-
LEK	-	-	0.833 (µg)	0.4 ± 0.55 ^
"	-	-	0.093 (µg)	15.5 ± 1.46 ^
"	-	-	0.010 (µg)	42.0 ± 2.05 ^
"	-	-	0.001 (µg)	63.2 ± 1.98 ^
SP-AAP	1	0.123 ± 0.018	0.5	2.5 ± 0.32 ^
"	0.1	0.157 ± 0.021	0.05	14.6 ± 1.79 ^
"	0.01	0.112 ± 0.001 ^	0.005	54.4 ± 0.99 ^
"	0.001	0.191 ± 0.093		
SP-C1	0.32	0.272 ± 0.038 ^^	0.16	50.0 ± 3.37 ^
"	0.032	0.101 ± 0.020	0.016	78.2 ± 3.82
"	0.0032	0.146 ± 0.038	0.0016	83.3 ± 1.28 ^^
"	0.0003	0.024 ± 0.002		
SP-C2	1	0.097 ± 0.006 ^^	0.5	3.0 ± 0.55 ^
"	0.1	0.114 ± 0.016	0.05	38.7 ± 2.86 ^
"	0.01	0.239 ± 0.018 ^	0.005	72.4 ± 2.29 ^
"	0.001	0.289 ± 0.072		
SP-C3	0.5	0.064 ± 0.006	0.25	0 ^
"	0.05	0.107 ± 0.021	0.025	9.8 ± 1.18 ^
"	0.005	0.178 ± 0.023 ^^	0.0025	36.4 ± 2.01 ^
"	0.0005	0.230 ± 0.032 ^^		
SP-C4	0.1	0.047 ± 0.010	0.5 (ß+)	0.4 ± 0.78 ^
"	0.01	0.114 ± 0.013	0.05	5.9 ± 0.81 ^
"	0.001	0.139 ± 0.055	0.005	40.2 ± 2.24 ^
"	0.0001	0.447 ± 0.269		
SP-C5	0.5	0.094 ± 0.026	0.5	13.4 ± 1.08 ^
"	0.05	0.113 ± 0.023	0.05	42.9 ± 2.81 ^
"	0.005	0.165 ± 0.055	0.005	40.8 ± 2.46 ^
"	0.0005	0.262 ± 0.039 ^		

Values represent mean ± S.E.M. of triplicate determinations.
LEK : Leu-enkephalin
^ : $p < 0.001$ compared with control or buffer
^^ : $p < 0.005$ compared with control or buffer
(ß+) : Most potent fraction in ß-endorphin RIA

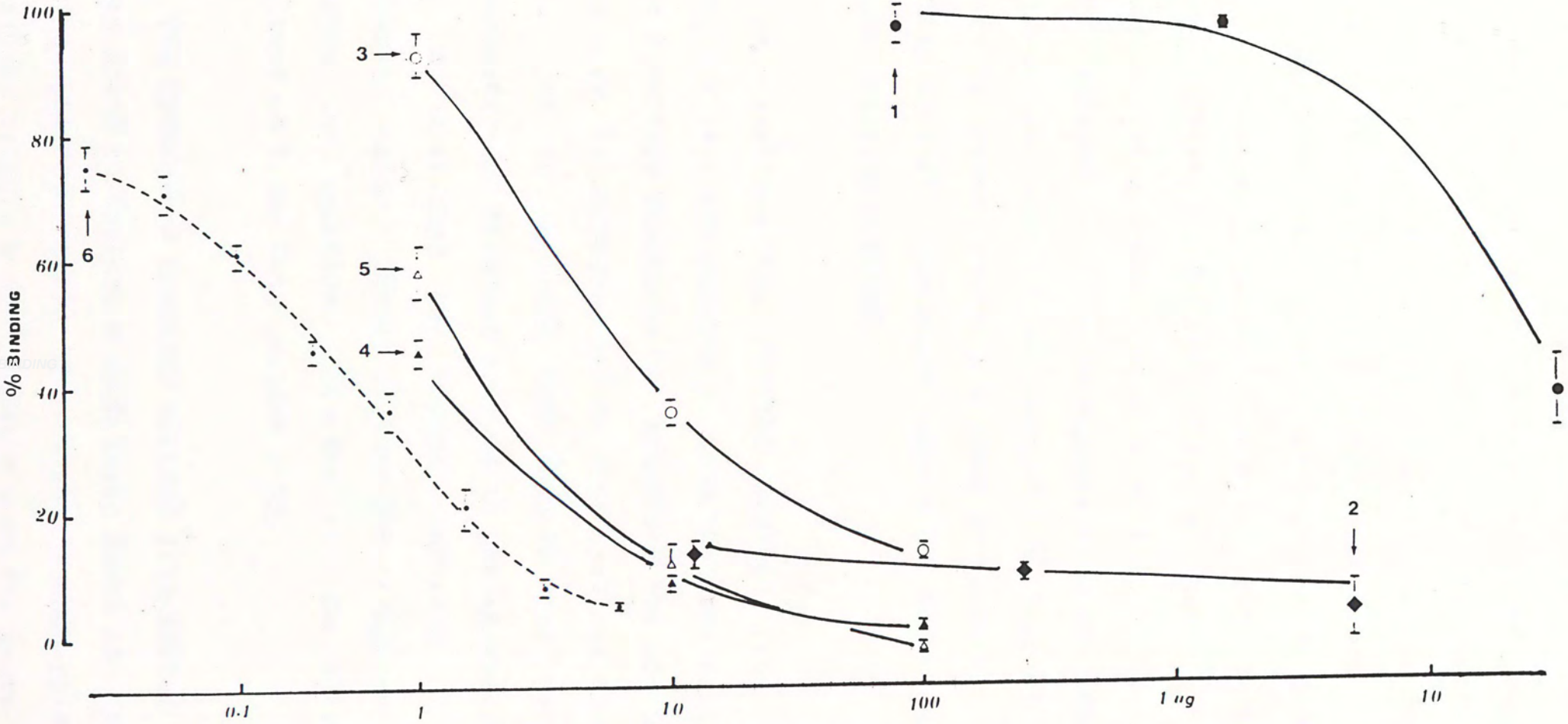
Figure 6-8

Displacement of [^{125}I]ß-endorphin from ß-endorphin antiserum by the fractions of CM-cellulose derived from snake pituitary

Labels on the figure :

1 : SP-C1
2 : SP-C2
3 : SP-C3
4 : SP-C4
5 : SP-C5

6 : Standard ß-endorphin (human)


100
80
60
40
20
0
% BINDING
1
2
3
4
5
6
0.1
1
10
100
1 ng
10
Snake Pituitary fraction from CM-cellulose
(ng)

fractions mentioned previously (table 6-4). The case is also true when considering the β-endorphin-like immunoreactivity (figure 6-8) of the fractions derived from CM-cellulose. The presence of most of the activities in the adsorbed fractions is similar to the results on mammalian species. Fraction SP-1-C4 exhibited the highest and fraction SP-1-C1 the least, opiate receptor binding activity and β-endorphin-like immunoreactivity. Fraction SP-1-C5 had the next highest immunoreactivity and fraction SP-1-C3 had lower still immunoreactivity. Thus it appears that the immunoreactivity of these two latter fractions did not completely correlate with their opiate receptor binding activities. In contrast, fraction SP-1-C2 possessed both high immunoreactivity and receptor binding activity.

In examining the results obtained from steroidogenic assay, it is a bit awkward to find that the abilities of the snake pituitary fractions to stimulate the adrenal decapsular cells seem to increase as the dose used was decreased (table 6-4). It is believed that this is due to too high a concentration of fraction applied in the incubation medium and thus it disturbed the normal functions of the adrenal decapsular cells. Hence another dose response test was done to show the relative potencies of the snake pituitary fractions at lower dose (figure 6-9).

The ConA-Ultra fraction derived from method II (figure 6-6) was found to contain a much lower level of opiate receptor binding activity (table 6-5) and β-endorphin-like immunoreactivity (figure 6-10) compared with the snake pituitary AAP

Figure 6-9 Dose response curves of snake and lamprey pituitary fractions

Standard curve :

Standard	Dose (nM)	Steroidogenic activity (pg corticosterone/hr/25,000 cells)
Control	0	0.001 ± 0.001
ACTH	10	31.130 ± 3.268
"	5	25.636 ± 3.664
"	2.5	25.499 ± 1.358
"	1.25	9.409 ± 2.095
"	0.625	5.002 ± 0.325
"	0.3125	0.758 ± 0.442

Values represent mean ± S.E.M. of triplicate determinations.

Labels on the figure :

(1) Snake pituitary fraction: SP-C3
(2) Snake pituitary AAP
(3) Snake pituitary fraction: SP-C2
(4) Snake pituitary fraction: SP-C1
(5) Lamprey pituitary AAP [discussed in Chapter 8]

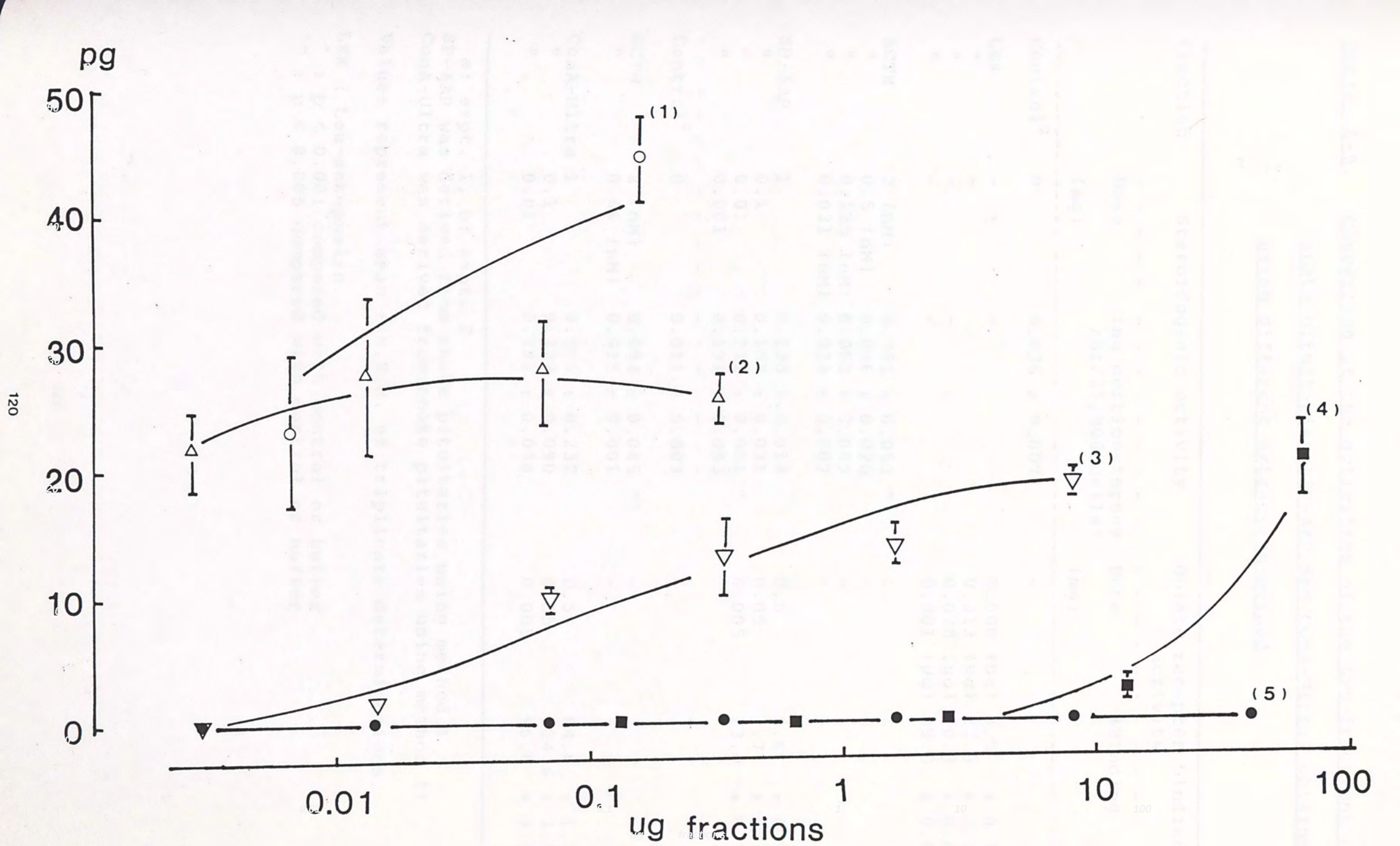

pg
50
40
30
20
10
0
(1)
(2)
(3)
(4)
(5)
0.01
0.1
1
10
100
ug fractions

Table 6-5. Comparison of the activities of the two fractions of snake pituitaries (SP-AAP and ConA-Ultra) obtained using different extraction method

Fraction	Steroidogenic activity		Opiate receptor binding activity	
	Dose (mg)	(ng corticosterone /hr/25,000 cells)	Dose (mg)	%Binding
Control[a]	0	0.036 ± 0.007	-	-
LEK	-	-	5.000 (µg)	4.5 ± 0.12 ^
"	-	-	0.313 (µg)	22.9 ± 3.14 ^
"	-	-	0.020 (µg)	70.3 ± 0.61 ^
"	-	-	0.001 (µg)	89.0 ± 0.85
ACTH	2 (nM)	0.351 ± 0.053 ^^	-	-
"	0.5 (nM)	0.096 ± 0.020	-	-
"	0.125 (nM)	0.052 ± 0.009	-	-
"	0.031 (nM)	0.028 ± 0.002	-	-
SP-AAP	1	0.123 ± 0.018	0.5	5.6 ± 0.95 ^
"	0.1	0.157 ± 0.021	0.05	1.7 ± 0.39 ^
"	0.01	0.112 ± 0.001 ^	0.005	13.0 ± 0.79 ^
"	0.001	0.191 ± 0.093		
Control[b]	0	0.011 ± 0.003	-	-
ACTH	2 (nM)	0.654 ± 0.085 ^^	-	-
"	0.66 (nM)	0.015 ± 0.001	-	-
ConA-Ultra	1	0.305 ± 0.232	0.5	84.6 ± 1.33 ^^
"	0.1	0.322 ± 0.090	0.05	104.6 ± 3.14
"	0.01	0.282 ± 0.038	0.005	96.0 ± 2.10

a: expt. 1, b: expt. 2
SP-AAP was derived from snake pituitaries using method I
ConA-Ultra was derived from snake pituitaries using method II

Values represent mean ± S.E.M. of triplicate determinations.

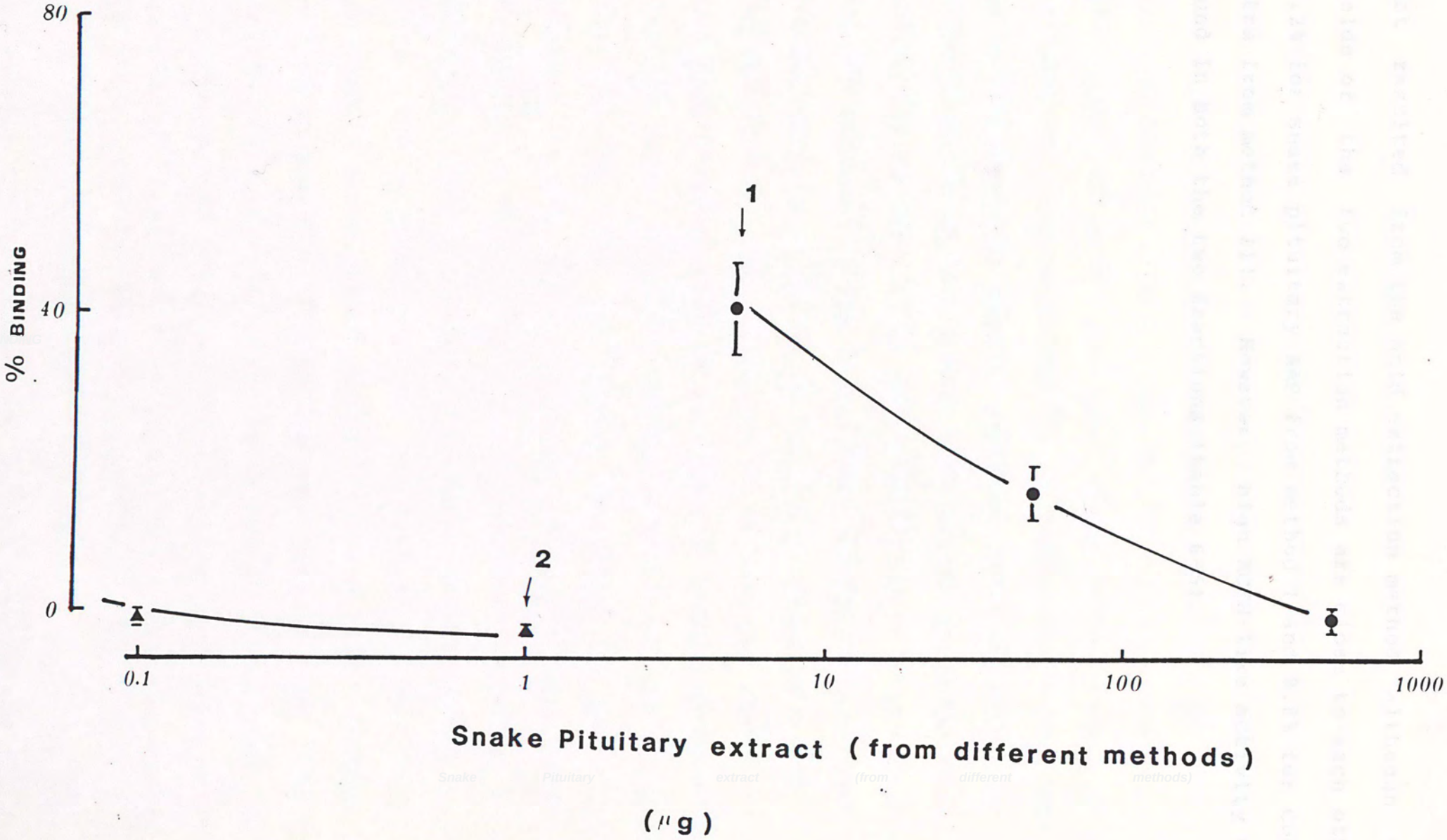
LEK : Leu-enkephalin
^ : $p < 0.001$ compared with control or buffer
^^ : $p < 0.005$ compared with control or buffer

Figure 6-10

Displacement of [^{125}I]β-endorphin from β-endorphin antiserum by snake pituitary AAP (SP) derived from method I and the fraction, ConA-Ultra, derived from method II

Labels on the figure :

1 : ConA-Ultra
2 : Snake pituitary AAP


80
40
0
% BINDING
1
2
0.1
1
10
100
1000
Snake Pituitary extract (from different methods)
(μg)

that resulted from the acid extraction method although the yields of the two extraction methods are close to each other (8.2% for snake pituitary AAP from method I and 8.8% for ConA-Ultra from method II). However, high ACTH-like activity was found in both the two fractions (table 6-5).

6.3.4 Discussion - snake pituitary

Both methods resulted in a high yield of ACTH activity but opiate activities (involving binding to receptor and antiserum respectively) were high only in snake pituitary AAP which was derived from the method I. This suggests a loss of opiate-like activity from the snake pituitaries in the purification step in method II, probably due to the loss in low molecular weight opioid peptides such as met- and leu-enkephalins which have a molecular weight below 1000. The possibility of the loss of opiate activity to be due to sodium ions was excluded by the result that the opiate-like materials were adsorbed in CM-cellulose and the presence of β-endorphin-like immunoreactivity. However, it is believed that both ACTH and β-endorphin will not be lost in this method as they have a molecular weight over 1000 and below 10,000 (table 6-6). Then these results seem to imply a relatively low level of β-endorphin in the snake pituitary compared with ACTH and enkephalins. This prediction is indirectly supported by the observation that β-endorphin immunoreactivity found in ConA-Ultra, the fraction derived from method II, was much lower than that in snake pituitary AAP (figure 6-10). Whether the loss of β-enodrphin-like immunoreactivity in the ConA-Ultra fraction derived from method II is due to the degradation of the opiate-like material by specific protease which is expected to have been denatured in the heating procedure in method I remains to be elucidated.

Results from the fractions derived from method I indicate

Table 6-6 Molecular weights of various opioid peptides and ACTH

Hormone	Molecular weights
MW > 10,000	
POMC	~30,000
MW > 1,000	
ACTH	4,500
β-endorphin	3,500
Dynorphin(1-17)	1,750
MW < 1,000	
Leu-enkephalin	555.7
Met-enkephalin	573.8

that, there are high levels of ACTH- and endorphin-like activities in the snake pituitary. Furthermore, the endorphin-like materials were also found to be active in displacing DADLE from rat brain membranes. The result that most of the activities were found in the adsorbed fractions eluted from CM-cellulose is similar to the results from mammalian pituitaries and the snake brain. However, the presence of all these activities in the snake pituitary is somewhat predictable since the pituitary gland is long known to be the tissue that secretes ACTH and β-endorphin in mammalian species. The co-existence of high levels of both ACTH- and endorphin-like activities implies an importance of these hormones in the snake pituitary. It is believed that the functions of these hormones in the snake is similar to their counterparts in higher vertebrates. However, whether they are also derived from POMC as in mammals remains to be studied in the future.

6.4 Turtle heart

6.4.1 Introduction - turtle heart

Although ACTH and opiates exert profound functions in mammalian hearts (section 4.1), their actions on the hearts of reptiles still remained a question although there exists a complete reptilian pituitary-adrenal axis similar to that of birds and mammals (Lofts, 1978; Callard and Callard, 1978). Presence of ACTH and opiates in the heart of reptiles was not available in the literature.

ACTH and opiate activities in the hearts of various mammals were discussed in chapter 4. In this section, the result from the heart of a reptile is presented as a comparison.

6.4.2 Materials and methods - extraction of turtle heart

The extraction protocol is shown in figure 6-11. Acid acetone powder (AAP) was prepared from acetone powder (AP) of turtle heart as described in section 4.2.2.1. The AAP obtained was then loaded on Sephadex G-25 and eluted with 0.1 N acetic acid (figure 6-12). After that, the first fraction which had a high enough yield was subjected on a CM-cellulose column which was then eluted with stepwise gradients of ammonium acetate. Finally, a solution of 0.6 N ammonium hydroxide was used to strip any material stongly adsorbed on the column (figure 6-13).

Figure 6-11 Extraction and purification of ACTH-like and opiate-like activities from turtle heart

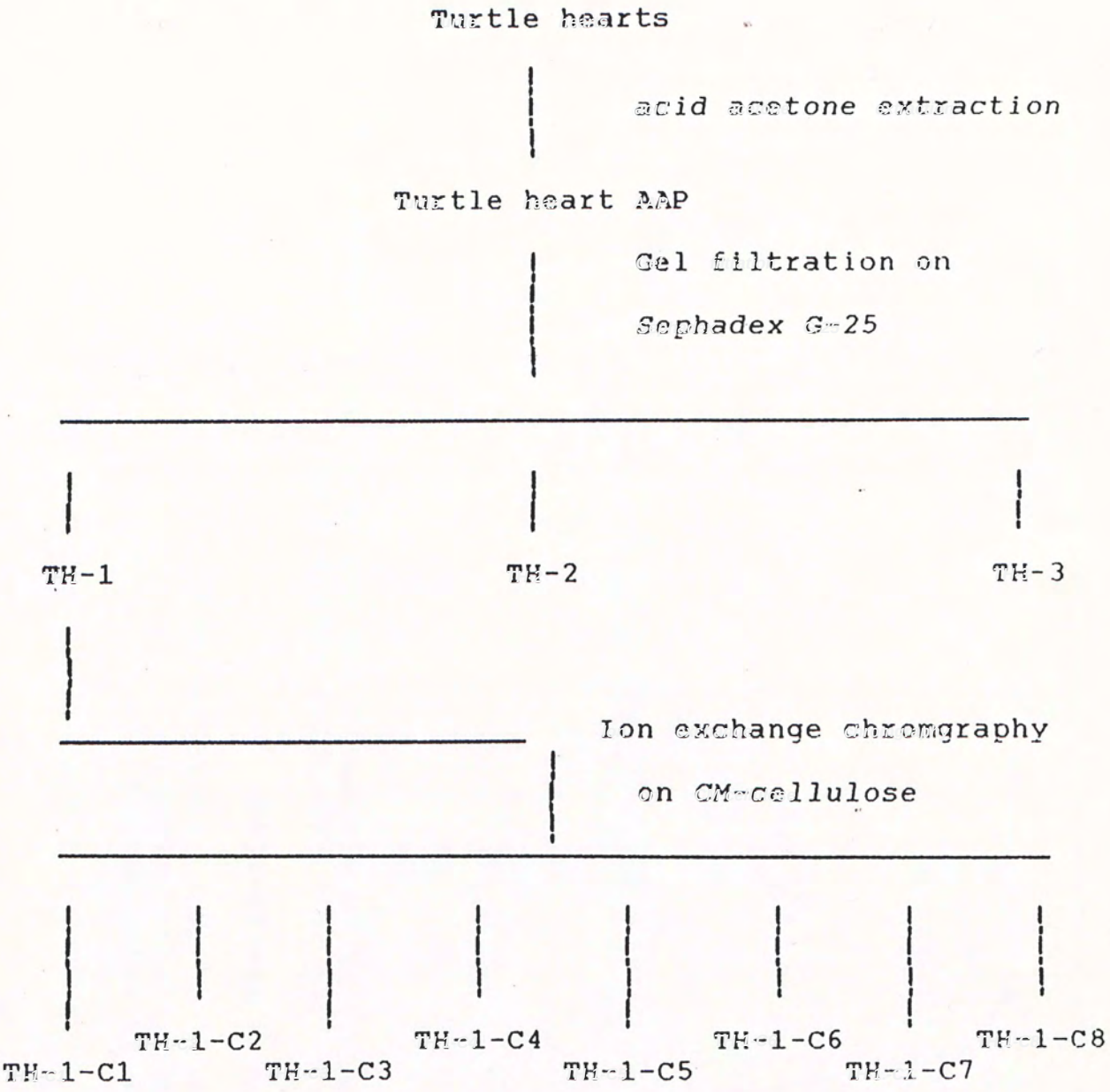


Figure 6-12

Gel filtration of turtle heart AAP (60 mg) on a Sephadex G-25 column (3.5 x 80 cm). Fraction size = 5 ml.

Buffer: 0.1 M acetic acid.

Yields: TH-1, 50 mg; (Void volume)
TH-2, trace amount;
TH-3, trace amount.

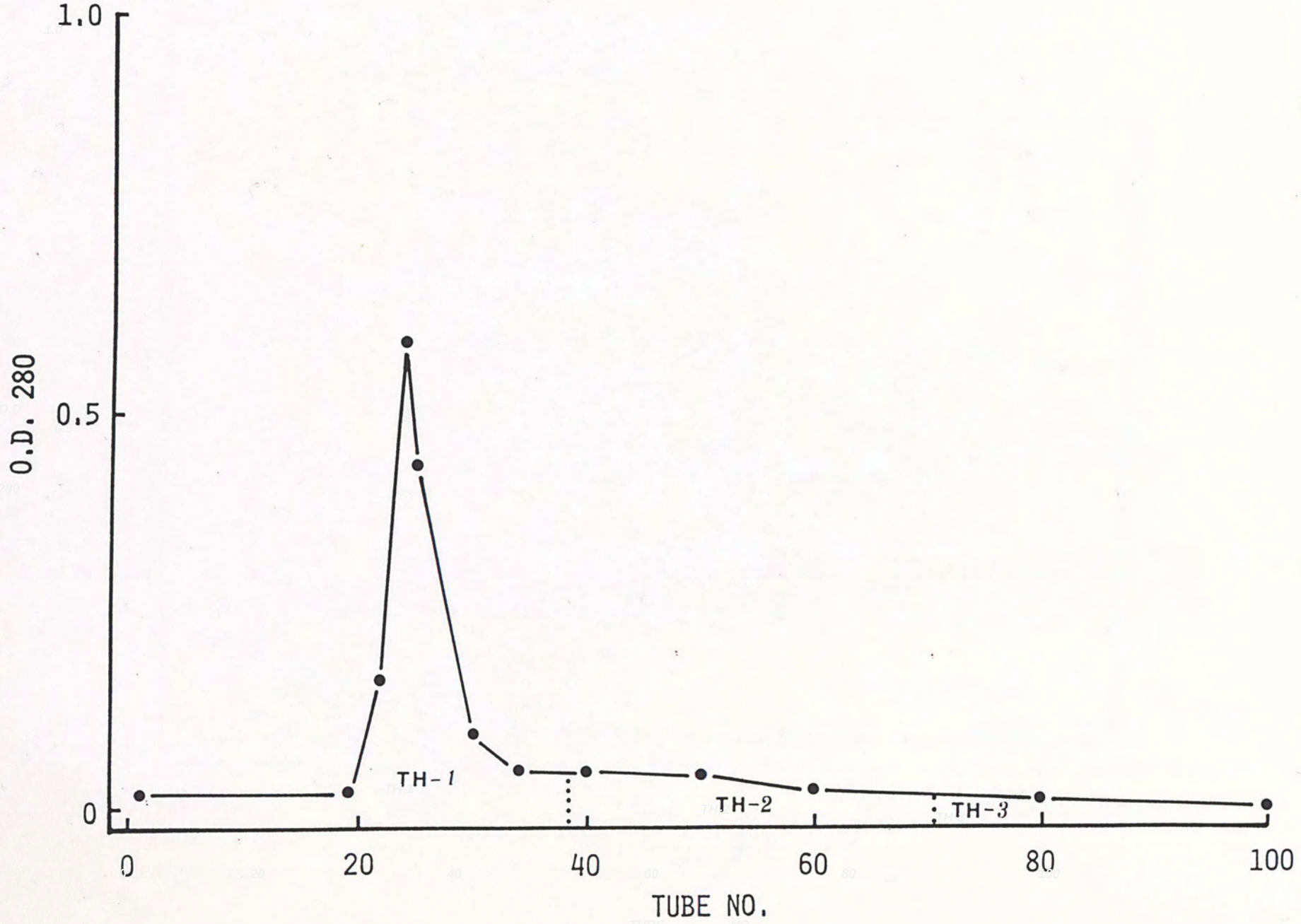

1.0
0.5
0
O.D. 280
TH-1
TH-2
TH-3
0
20
40
60
80
100
TUBE NO.

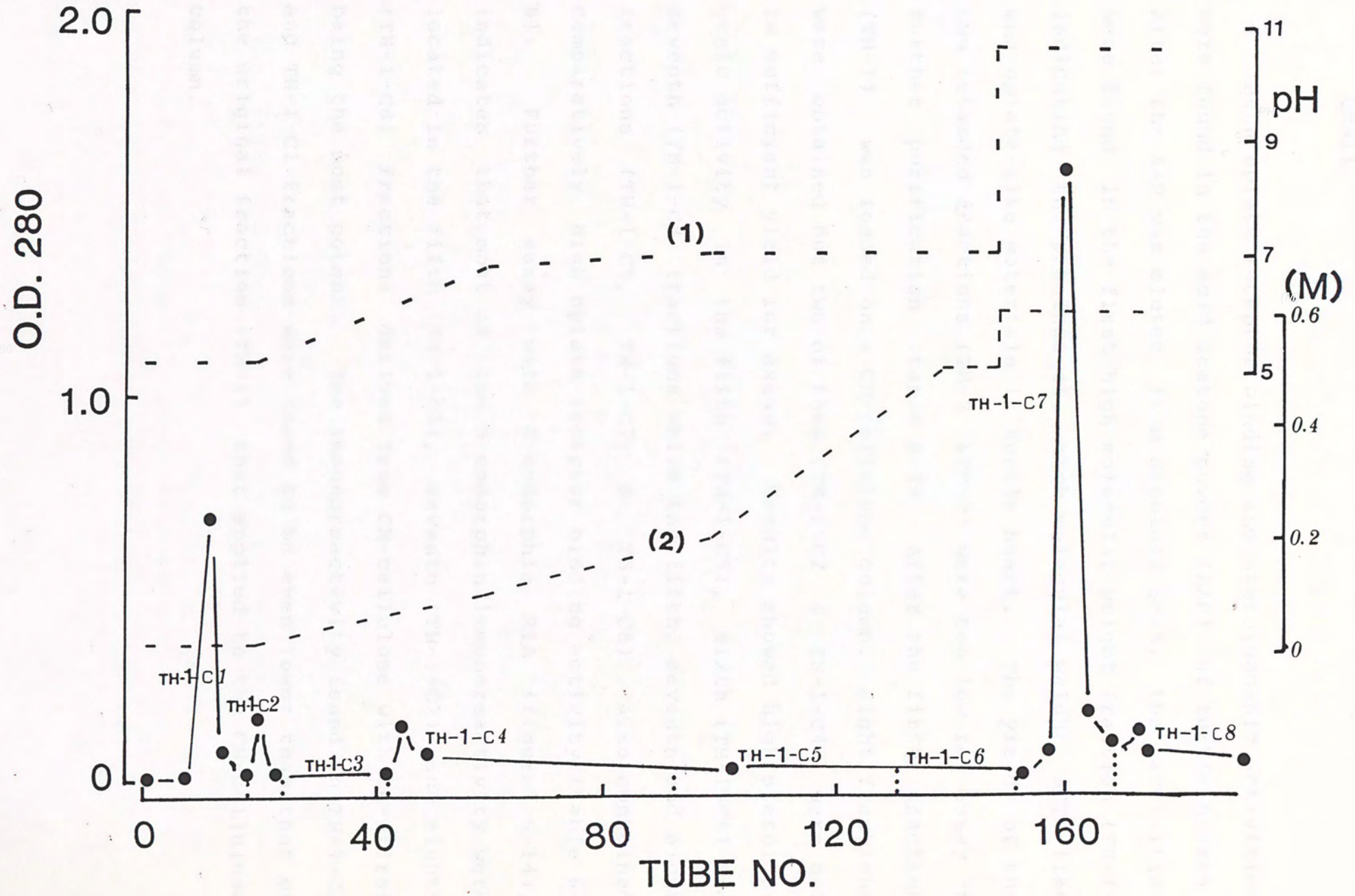
Figure 6-13

Ion exchange chromatography of turtle heart fraction TH-1 on a CM-cellulose column (1.4 x 36 cm). The fraction (129.4 mg) was dissolved in starting buffer, centrifuged and the resulting supernatant was applied on the column. Fraction size = 5 ml.

Buffer: 10 mM NH_4OAc, pH 4.6 (fractions 1-20);
10 - 100 mM NH_4OAc, pH 4.6 - 6.7 (fractions 21-60);
0.1 - 0.2 M NH_4OAc, pH 6.7 - 7 (fractions 61-100);
0.2 - 0.5 M NH_4OAc, pH 7 (fractions 101-140);
0.5 M NH_4OAc, pH 7 (fractions 141-155);
0.6 M NH_4OH, pH 10.6; (fractions 156-180).

Yields: TH-1-C1, 25.5 mg;
TH-1-C2, trace amount;
TH-1-C3, trace amount;
TH-1-C4, trace amount;
TH-1-C5, 6 mg;
TH-1-C6, 2 mg;
TH-1-C7, 4.9 mg;
TH-1-C8, 2.6 mg.

(1) : pH gradient
(2) : ionic gradient
(M) : Molarity of eluent


2.0
1.0
0
O.D. 280
11
9
7
5
pH
(M)
0.6
0.4
0.2
0
(1)
(2)
TH-1-C1
TH-1-C2
TH-1-C3
TH-1-C4
TH-1-C5
TH-1-C6
TH-1-C7
TH-1-C8
0
40
80
120
160
TUBE NO.

6.4.3 Results : ACTH-like and opiate-like activities in turtle heart

Both opiate receptor binding and steroidogenic activities were found in the acid acetone powder (AAP) of turtle hearts. After the AAP was eluted from Sephadex G-25, the activities were found in the first high molecular weight fraction (TH-1) indicating the presence of high molecular weight ACTH-like and opiate-like materials in turtle heart. The yields of the two retarded fractions (TH-2 &TH-3) were too low for assay or further purification (table 6-7). After the first fraction (TH-1) was loaded on a CM-cellulose column, eight fractions were obtained but two of them (TH-1-C2 & TH-1-C4) were not in sufficient yield for assay. Results showed high steroidogenic activity in the fifth (TH-1-C5), sixth (TH-1-C6) and seventh (TH-1-C8) fractions while the fifth, seventh and eight fractions (TH-1-C5, TH-1-C7 & TH-1-C8) also contained comparatively high opiate receptor binding activity (table 6-8). Further assay with β-endorphin RIA (figure 6-14), indicates that most of the β-endorphin immunoreactivity were located in the fifth (TH-1-C5), seventh (TH-1-C7) and eighth (TH-1-C8) fractions derived from CM-cellulose with the first being the most potent. The immunoreactivity found in TH-1-C3 and TH-1-C1 fractions were found to be even lower than that of the original fraction (TH-1) that applied to the CM-cellulose column.

Table 6-7. Steroidogenic and opiate activities of turtle heart fractions eluted from Sephadex G-25

Fraction	Steroidogenic activity		Opiate receptor binding activity	
	Dose (mg)	(ng corticosterone /hr/25,000 cells)	Dose (mg)	%Binding
Control[a]	0	0.028 ± 0.008	-	-
ACTH	1 (nM)	1.511 ± 0.082 ^	-	-
"	0.111 (nM)	0.551 ± 0.036 ^	-	-
"	0.012 (nM)	0.080 ± 0.027	-	-
LEK	-	-	4 (µg)	16.8 ± 1.34 ^
"	-	-	0.8 (µg)	38.1 ± 3.10 ^
"	-	-	0.16 (µg)	79.0 ± 2.76 ^
"	-	-	0.032 (µg)	104.9 ± 2.99
TH-AAP	4	0.294 ± 0.020 ^	0.5	59.7 ± 8.75
"	0.4	0.030 ± 0.012	0.05	108.3 ± 3.92
Control[b]	0	0.007 ± 0.000	-	-
ACTH	1 (nM)	1.135 ± 0.193 ^^	-	-
"	0.111 (nM)	0.239 ± 0.038 ^^	-	-
"	0.012 (nM)	0.009 ± 0.001	-	-
TH-1	1	0.145 ± 0.031	0.5	45.6 ± 2.98 ^
"	0.01	0.012 ± 0.002	0.05	86.2 ± 4.46
"	0.001	0.003 ± 0.000		
TH-2	trace	ND		ND
TH-3	trace	ND		ND

a: Expt. 1, b: Expt. 2

Values represent mean ± S.E.M. of triplicate determinations.

LEK : Leu-enkephalin
ND : Not determined due to insufficient material
^ : $p < 0.001$ compared with control or buffer
^^ : $p < 0.005$ compared with control or buffer

Table 6-8. Steroidogenic and opiate receptor binding activities of CM-cellulose fractions derived from turtle heart fraction: TH-1

Fraction	Steroidogenic activity		Opiate receptor binding activity	
	Dose (mg)	(ng corticosterone /hr/25,000 cells)	Dose (mg)	%Binding
Control	0	0.011 ± 0.003	-	-
ACTH	2 (nM)	0.654 ± 0.085 ^^	-	-
"	0.2 (nM)	0.003 ± 0.001	-	-
LEK	-	-	0.313 (μg)	22.9 ± 3.14 ^
"	-	-	0.078 (μg)	52.9 ± 1.37 ^
"	-	-	0.020 (μg)	70.3 ± 0.61 ^
"	-	-	0.005 (μg)	80.4 ± 1.58 ^
TH-1	1	0.155 ± 0.019 ^^	0.5	71.4 ± 2.24
"	0.1	0.008 ± 0.003	0.05	95.9 ± 2.27
"	0.01	0.005 ± 0.001	0.005	99.6 ± 1.43
TH-1-C1	1	0.657 ± 0.656	0.1	102.4 ± 1.03
"	0.1	0.005 ± 0.001	0.01	104.8 ± 0.83
"	0.01	UD	0.001	103.7 ± 2.78
TH-1-C2	trace	ND		ND
TH-1-C3	1	0.013 ± 0.001	0.5	87.2 ± 1.89
"	0.1	0.009 ± 0.002	0.05	104.7 ± 0.61
"	0.01	0.008 ± 0.003	0.005	98.3 ± 0.88
TH-1-C4	trace	ND		ND
TH-1-C5	1	0.346 ± 0.031 ^	0.25 (β+)	72.0 ± 2.34 ^
"	0.1	0.014 ± 0.002	0.025	91.6 ± 2.52
"	0.01	0.016 ± 0.003	0.0025	93.6 ± 2.13
TH-1-C6	1	0.551 ± 0.042 ^	0.1	85.9 ± 3.12
"	0.1	0.618 ± 0.140	0.01	102.0 ± 4.50
"	0.01	0.016 ± 0.001	0.001	100.0 ± 0.86
TH-1-C7	1	0.471 ± 0.074 ^^	0.5	71.2 ± 0.75 ^
"	0.1	0.183 ± 0.010 ^	0.05	102.2 ± 2.08
"	0.01	0.013 ± 0.001	0.005	100.4 ± 1.84
TH-1-C8	1	0.069 ± 0.004 ^	0.2	74.5 ± 1.63 ^
"	0.1	0.013 ± 0.002	0.02	94.3 ± 2.27
"	0.01	0.011 ± 0.001	0.002	99.6 ± 1.43

Values represent mean ± S.E.M. of triplicate determinations.
LEK : Leu-enkephalin
UD : Undetectable
ND : Not determined due to insufficient material
^ : $p < 0.001$ compared with control or buffer
^^ : $p < 0.005$ compared with control or buffer
(β+) : Most potent fraction in [illegible] RI

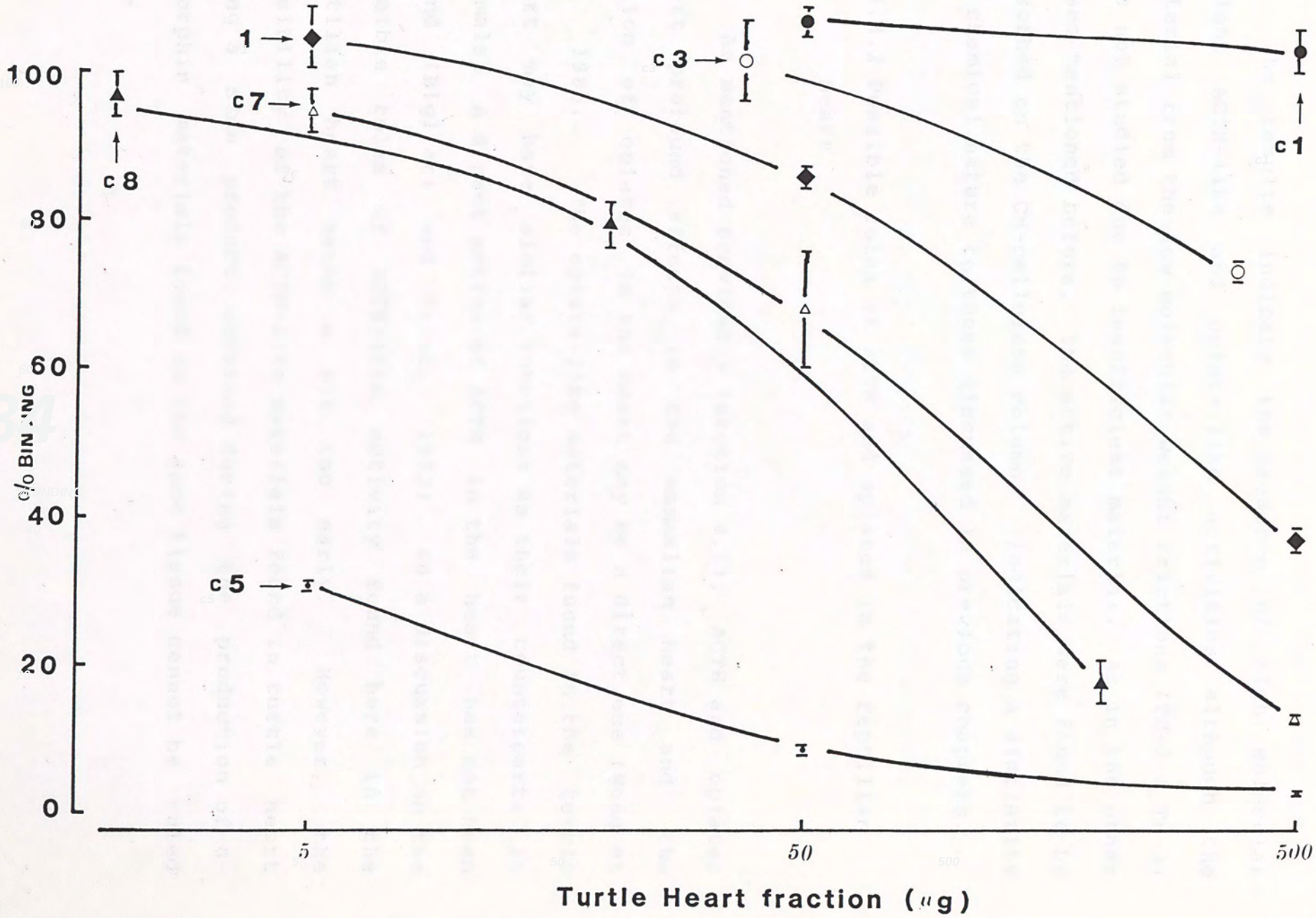
Figure 6-14

Displacement of ^{125}I ß-endorphin from ß-endorphin antiserum by the fractions of CM-cellulose derived from the first fraction (TH-1) of Sephadex G-25 of turtle heart

Label on the figure :

1 : TH-1

c1 : TH-1-C1
c3 : TH-1-C3
c5 : TH-1-C5
c7 : TH-1-C7
c8 : TH-1-C8


1
c 3
c 7
c 8
c 1
c 5
100
80
60
40
20
0
% BINDING
5
50
500
Turtle Heart fraction (µg)

6.4.4 Discussion - turtle hearts

6.4.4.1 About the results

The results indicate the presence of high molecular weight ACTH-like and opiate-like activities although the material from the low molecular weight fractions (TH-2 & TH-3) was not studied due to insufficient material. As in the other cases mentioned before, the active materials were found to be adsorbed on the CM-cellulose column, indicating a similarity in chemical nature to those discussed in previous chapters.

6.4.4.2 Possible roles of ACTH and opiates in the reptilian heart

As mentioned previously (section 4.1), ACTH and opiates exert profound effects in the mammalian heart and the action of opiates in the heart may be a direct one (Wong et al, 1985). The opiate-like materials found in the turtle heart may have similar functions as their counterparts in mammals. A direct action of ACTH in the heart has not been found (Biglieri and Kater, 1983) so a discussion on the possible roles of ACTH-like activity found here in the reptilian heart seems a bit too early. However, the possibility of the ACTH-like materials found in turtle heart being a side product obtained during the production of β-endorphin materials found in the same tissue cannot be ruled out.

6.4.4.3 Mammalian and reptilian hearts

Comparing the results with those obtained from the hearts (section 4.1) of rodents, both types of active materials were found to be adsorbed on the CM-cellulose and eluted in similar positions. Then it seems quite logical to suggest that ACTH- and opiate-like activities are universal in the hearts of mammals as well as reptiles. The presence of high molecular weight fractions that possessing both opiate receptor binding, steroidogenic and β-endorphin-like activities in both turtle hearts and hamster hearts indicate that both ACTH and opiates have long been a component of heart tissue before the mammals have been evolved from reptiles.

6.5 Turtle intestine

6.5.1 Introduction - turtle intestine

The presence of endogenous opioid peptides in the guts of various mammalian species has been studied extensively. Enkephalin in the guts of porcine (Alumets et al, 1983), guinea pig, (Musacchio et al, 1980), rat (Wang et al, 1984) and human (Ferri et al, 1987); dynorphin (Kromer etr al, 1981) in the guts of guinea pig and rat (Spampinato and Goldstein, 1983); β-endorphin in human (Sjolund et al, 1983) and in rat (Orwoll and Kendall, 1980) were reported. ACTH was also found in the rat gut (Orwoll and Kendall, 1980; Sanchez-Franco et al, 1981; Saito et al, 1983) by some groups.

No studies in opiates and ACTH in the reptilian gut have appeared. So, the turtle intestine was studied in this thesis to see whether ACTH-like and opiate-like activities exist in the reptilian gut.

6.5.2 Materials and methods - extraction of turtle intestine

The method used to extract turtle intestine was similar to that used for the turtle heart (figure 6-15). Turtle intestine acid acetone powder (AAP) was prepared from its acetone powder (AP) as described in section 4.2.2.1. The AAP was then loaded on Sephadex G-25 and eluted with 0.1 N acetic acid (figure 6-16). After that, the first fraction was

Figure 6-15 Extraction and purification of ACTH-like and opiate-like activities from turtle intestine

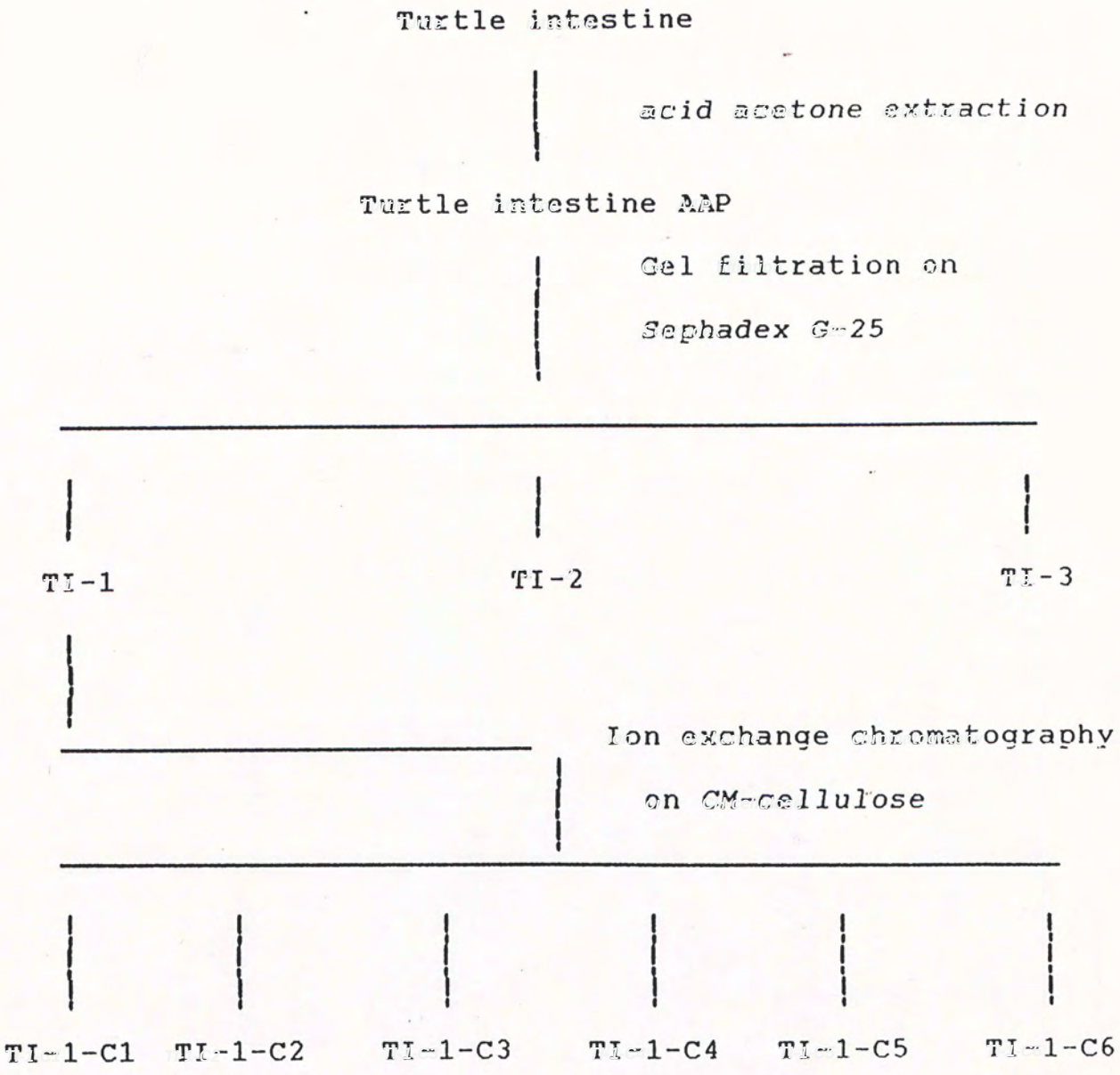
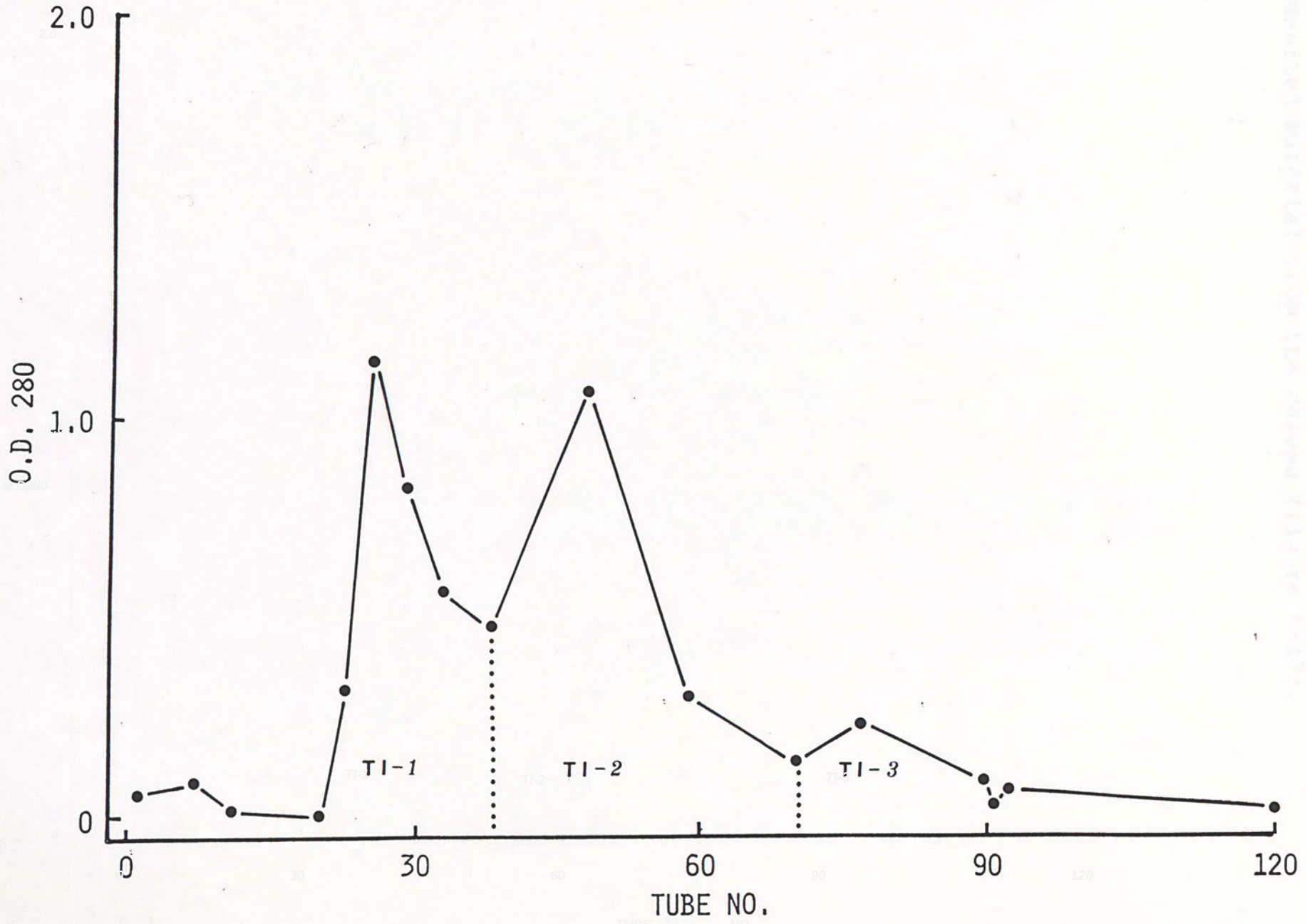


Figure 6-16

Gel filtration of turtle intestine AAP on a Sephadex G-25 column (3.5 x 80 cm). The AAP (413 mg) was dissolved in buffer, centrifuged and the supernatant was then applied on the column. Fraction size = 5 ml.

Buffer: 0.1 M acetic acid.

Yields: TI-1, 88.5 mg; (Void volume)
TI-2, 140 mg;
TI-3, 5 mg.


2.0
1.0
0
O.D. 280
TI-1
TI-2
TI-3
0
30
60
90
120
TUBE NO.

chromatographed on a CM-cellulose column using stepwise gradient elution with ammonium acetate. Finally, a solution of 0.6 N ammonium hydroxide was used to recover any strongly adsorbed material from the column (figure 6-17).

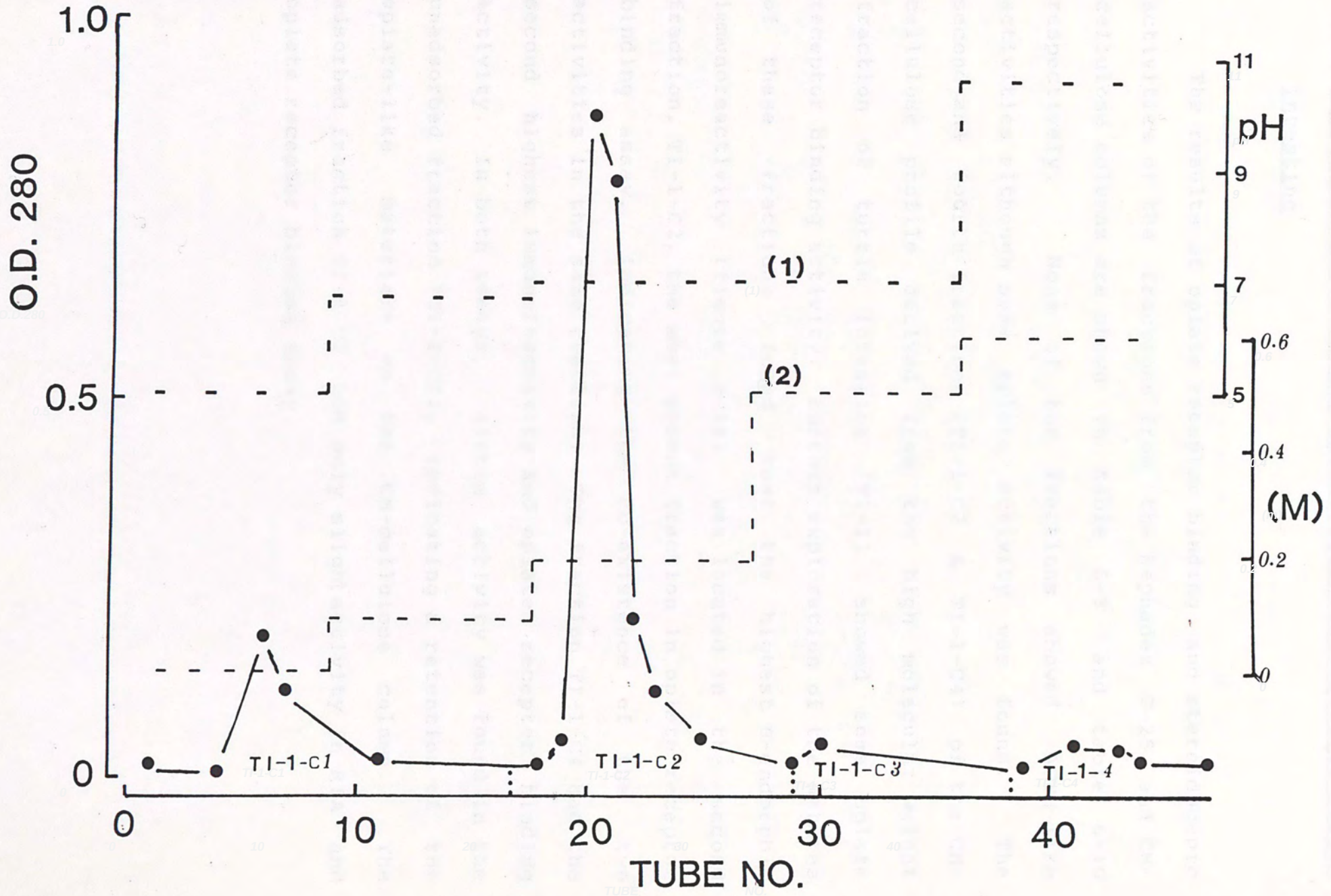
Figure 6-17 Turtle intestine fraction TI-1 on CM-cellulose

Ion exchange chromatography of turtle intestine fraction TI-1 (50 mg) on on CM-cellulose (1.2 x 17 cm). Fraction size = 3 ml.

Buffer: 10 mM NH_4OAc, pH 4.6 (fractions 1-9);
100 mM NH_4OAc, pH 6.7 (fractions 10-18);
0.2 M NH_4OAc, pH 7 (fractions 19-27);
0.5 M NH_4OAc, pH 7 (fractions 28-36);
0.6 M NH_4OH, pH 10.6; (fractions 37-47).

Yields: TI-1-C1, 1.7 mg;
TI-C2, 9.7 mg;
TI-C3, 1 mg;
TI-C4, 8.3 mg.

(1) : pH gradient
(2) : ionic gradient
(M) : Molarity of eluent


1.0
0.5
0
O.D. 280
(1)
(2)
11
9
7
5
pH
0.6
0.4
0.2
0
(M)
TI-1-C1
TI-1-C2
TI-1-C3
TI-1-4
0
10
20
30
40
TUBE NO.

6.5.3 Results : ACTH-like and opiate-like activities of turtle intestine

The results of opiate receptor binding and steroidogenic activities of the fractions from the Sephadex G-25 and CM-cellulose columns are shown in table 6-9 and table 6-10 respectively. None of the fractions showed ACTH-like activities although some opiate activity was found. The second and fourth fractions (TI-1-C2 & TI-1-C4) of the CM-cellulose profile derived from the high molecular weight fraction of turtle intestine (TI-1) showed some opiate receptor binding activity. Further exploration of the natures of these fractions found that the highest β-endorphin immunoreactivity (figure 6-18) was located in the second fraction, TI-1-C2, the most potent fraction in opiate receptor binding assay, indicating the co-existence of the two activities in the same fraction. The fraction TI-1-C4 had the second highest immunoreactivity and opiate receptor binding activity. In both assays, little activity was found in the unadsorbed fraction (TI-1-C1), indicating a retention of the opiate-like materials on the CM-cellulose column. The adsorbed fraction TI-1-C3 had only slight activity in RIA and opiate receptor binding assay.

Table 6-9. Steroidogenic and opiate activities of turtle intestine fractions eluted from Sephadex G-25

Fraction	Steroidogenic activity		Opiate receptor binding activity	
	Dose (mg)	(ng corticosterone /hr/25,000 cells)	Dose (mg)	%Binding
Control[a]	0	0.090 ± 0.013	-	-
ACTH	1 (nM)	1.603 ± 0.072 ^	-	-
"	0.04 (nM)	0.140 ± 0.002	-	-
LEK	-	-	4 (µg)	16.8 ± 1.34 ^
"	-	-	0.8 (µg)	38.1 ± 3.10 ^
"	-	-	0.16 (µg)	79.0 ± 2.76 ^
"	-	-	0.032 (µg)	104.9 ± 2.99
TI-AAP	1	0.040 ± 0.006	0.5	60.9 ± 3.26 ^
"	0.1	0.028 ± 0.003	0.05	99.5 ± 2.33
"	0.01	0.009 ± 0.002		
Control[b]	0	0.024 ± 0.004	-	-
ACTH	1 (nM)	0.540 ± 0.067 ^^	-	-
"	0.5 (nM)	0.117 ± 0.029	-	-
"	0.25 (nM)	0.029 ± 0.006	-	-
TI-1	1	0.011 ± 0.006	0.5	63.0 ± 4.51 ^
"	0.1	0.014 ± 0.007	0.05	99.8 ± 0.73
"	0.01	0.032 ± 0.018	0.005	100.5 ± 4.40
TI-2	1	0.006 ± 0.002	0.5	82.4 ± 2.98
"	0.1	0.027 ± 0.003	0.05	100.0 ± 4.44
"	0.01	0.013 ± 0.001	0.005	109.5 ± 2.56
TI-3	1	0.032 ± 0.006	0.5	41.3 ± 4.97 ^
"	0.1	0.030 ± 0.004	0.05	98.2 ± 2.40
"	0.01	0.028 ± 0.005	0.005	95.3 ± 5.11

a: Expt. 1, b: Expt. 2

Values represent mean ± S.E.M. of triplicate determinations.

LEK : Leu-enkephalin
^ : $p < 0.001$ compared with control or buffer
^^ : $p < 0.005$ compared with control or buffer

Table 6-10. Steroidogenic and opiate activities of CM-cellulose fractions derived from turtle intestine fraction: TI-

Fraction	Steroidogenic activity		Opiate receptor binding activity		
	Dose (mg)	(ng corticosterone /hr/25,000 cells)	Dose (mg)	%Binding	
Control	0	0.036 ± 0.007	-	-	
ACTH	2 (nM)	0.351 ± 0.035 ^	-	-	
"	0.5 (nM)	0.096 ± 0.020	-	-	
"	0.125 (nM)	0.052 ± 0.009			
"	0.031 (nM)	0.028 ± 0.002			
LEK	-	-	0.01 (µg)	42.0	± 2.05 ^
"	-	-	0.003 (µg)	52.8	± 2.06 ^
"	-	-	0.001 (µg)	63.2	± 1.98 ^
TI-1-C1	0.2	0.018 ± 0.001	0.5	82.2	± 3.40
"	0.02	0.029 ± 0.003	0.05	83.8	± 1.61
"	0.002	0.023 ± 0.001	0.005	85.3	± 4.10
TI-1-C2	1	0.014 ± 0.000	0.5 (β+)	52.3	± 1.75 ^
"	0.1	0.024 ± 0.002	0.05	75.4	± 4.42
"	0.01	0.019 ± 0.001	0.005	85.0	± 2.57
TI-1-C3	0.1	0.043 ± 0.024	0.25	84.9	± 1.34
"	0.01	0.030 ± 0.012	0.025	74.4	± 2.81 ^^
"	0.001	0.021 ± 0.001	0.0025	90.7	± 1.31 ^^
TI-1-C4	0.5	0.017 ± 0.001	0.1	64.2	± 5.58 ^^
"	0.1	0.112 ± 0.084	0.01	79.4	± 1.79 ^^
"	0.01	0.023 ± 0.001	0.001	81.1	± 4.75

Values represent mean ± S.E.M. of triplicate determinations.

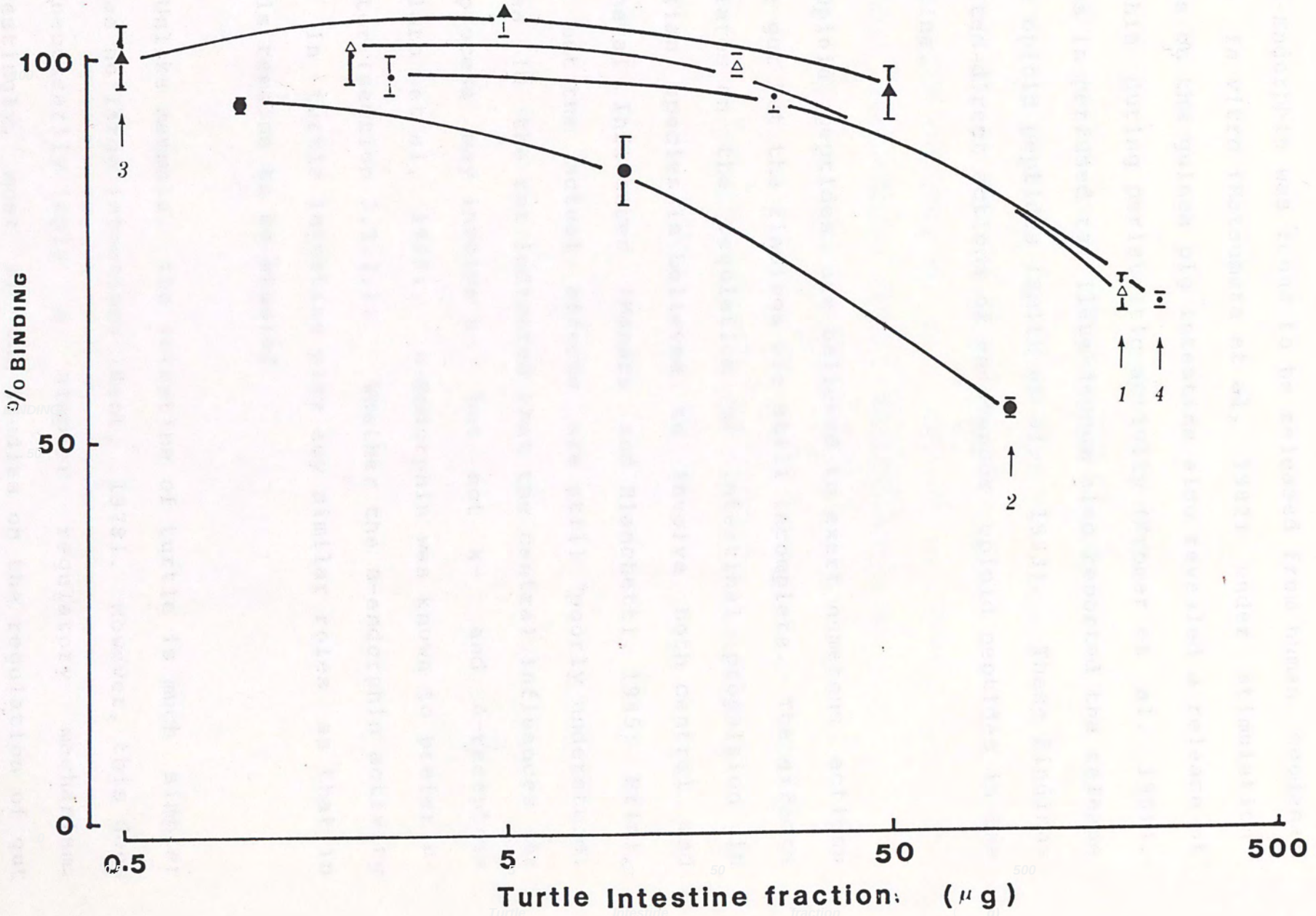
LEK : Leu-enkephalin
^ : $p < 0.001$ compared with control or buffer
^^ : $p < 0.005$ compared with control or buffer
(β+) : Most potent fraction in β-endorphin RIA

Figure 6-18

Displacement of ^{125}I-β-endorphin from β-endorphin antiserum by the fractions of CM-cellulose derived from the first fraction (TI-1) of Sephadex G-25 of turtle intestine

Labels on the figure :

1 : TI-1-C1
2 : TI-1-C2
3 : TI-1-C3
4 : TI-1-C4


100
50
0
% BINDING
3
2
1
4
0.5
5
50
500
Turtle Intestine fraction (μg)

6.5.4 Discussion - turtle intestine

ß-Endorphin was found to be released from human duodenal mucosa *in vitro* (Matsumura et al, 1982) under stimulation. Studies on the guinea pig intestine also revealed a release of dynorphin during peristaltic activity (Kromer et al, 1981). Studies in perfused rat ileum-jeunum also reported the release of the opioid peptides (Smith et al, 1983). These findings suggested direct actions of endogenous opioid peptides in the intestine.

Opioid peptides are believed to exert numerous actions on the gut but the findings are still incomplete. The effects of opiates on the regulation of intestinal propulsion in mammalian species is believed to involve both central and peripheral influences (Manara and Bianchetti, 1985; Primi, 1985) but the actual effects are still poorly understood. Studies in the rat indicated that the central influences of the process may involve μ- but not k- and δ-receptors (Parolaro et al, 1986). ß-Endorphin was known to prefer μ-receptor (section 3.2.3.2). Whether the ß-endorphin activity found in turtle intestine play any similar roles as that in mammals remains to be studied.

Unlike mammals, the intestine of turtle is much simpler and has no large intestines (Kent, 1978). However, this does not necessarily imply a simpler regulatory mechanism. Interestingly, most of the studies on the regulation of gut motility in reptiles were carried out on turtles but not on

other reptiles. Both excitatory and inhibitory actions of the vagus nerve have been reported (Skoczylas, 1978). However, the role of opiates found here in the turtle intestine remains to be studied in the future.

Furthermore, it is worth noting that met-enkephalin-like immunoreactivity was reported in gut tissue of vertebrates which are even more primitive than reptiles such as the eel (Elbal and Agulleiro, 1986) and the fish *Sparus auratus L.* (L'Hermite et al, 1985) while ACTH-like immunoreactivity was also reported recently in the gut of an invertebrate, the earthworm, (Kaloustian, 1986) indicating an ancient role of ACTH and opiates in the physiology of digestive system in the animal kingdom. However, the failure to detect ACTH-like material in the turtle intestine indicates that the level of ACTH-like material in turtle intestine is very low if present compared with that in mammals (section 6.3.1). However, whether this is true of the intestines of other reptiles and the role of ACTH in the reptilian digestive system remain to be clarified.

DATA AND RESULT FROM CLASS OSTEICHTHYES

– Grouper, flounder and eel

Chapter 7 DATA AND RESULT FROM CLASS OSTEICHTHYES - Grouper, Flounder and Eel

7.1 ACTH and opiates in fishes - an introduction

7.1.1 Teleosts are the most abundant fishes

Class Osteichthyes consists of the the higher bony fishes. In this chapter, three species of this class, flounder, eel, and the red grouper, *Epinephelus akaara*, from Infra class Teleostei were chosen for study. Teleosts expand and diversify in an elaborate adaptive radiation and up to now, there are more species of teleosts than there are of all other vertebrates combined. One has tried to name any function of other vertebrates which is not also performed by at least one teleost, including walking on land, flying, and maintaining a constant temperature (Webster and Webster, 1974a). However, teleosts are the most extensively studied among all the fishes so they were chosen for study in spite of their high species diversity.

7.1.2 ACTH and the teleosts

7.1.2.1 Presence of ACTH in the teleosts

The presence of ACTH-like and β-endorphin-like peptides in teleosts was studied most extensively in salmon. Although POMC-like immunoreactivity (Naito et al, 1984) has been

reported in pituitaries of different salmons, it was claimed that no ACTH molecule has been successfully purified from teleosts (Rodrigues and Sumpter, 1983). No successful puification of ACTH was recorded in the literature on teleosts up to 1987. Perhaps, the presence of ACTH-like bioactivity (Ng et al, 1987) in chinook salmon pituitary may give some hints to the presence of a true ACTH molecule. However, the results obtained from salmon can then serve as a standard guide for subsequent studies on teleosts.

7.1.2.2 Possible role of ACTH in teleosts

Before we can proceed to our results, one must first know that there is no adrenal gland in teleosts. An adrenocortical homologue called interrenal tissue was believed to be the peripheral target tissue of pituitary ACTH (Jorgensen, 1976) which copes with stress (Donaldson, 1981) in a mechanism independent of the activity of MSH (Pickering et al, 1986) which may, however, potentiate the process (Takahashi et al, 1985). Nevertheless, studies on trout confirmed the role of ACTH as the major factor in teleosts responding to stress although endorphin and α-MSH may also have some contributions to certain types of stress (Sumpter et al, 1985, 1986; Balm et al, 1987).

7.1.3 Opioid peptides and the teleosts

Two different endorphin molecules were discovered in the chum salmon pituitary (Takahashi et al, 1984) implying there may be other endorphin precursors in lower vertebrates

besides POMC. Opiate-like materials specific to different receptors (δ- and μ-) were found in the chinook salmon pituitary (Ng et al, 1987) and thus indirectly indicated the possible presence of endorphin and/or enkephalin (section 3.2.3.2). The role of endogenous opioid peptides in teleosts was still not well-known although an involvement of opiate in the reproductive cycle of catfish (Rosenblum, 1986) and stress response in trout (Sumpter et al, 1985) was found. Met-enkephalin was found to induce colour change in some species (Levina and Gordon, 1983) but not in others (Gordon and Levina, 1983) indicating that opiates may have different functions in different teleost species.

7.1.4 POMC of teleosts may be different from that of mammals

Discovery of more than one endorphin molecule in teleosts (Takahashi et al, 1984) suggested that POMC in teleosts may be quite different from its homologues in higher vertebrates. Furthermore, the regulatory mechanism of ACTH in teleosts is quite different from that of higher vertebrates. A significant factor that is involved is melanin concentrating hormone (MCH) which is a cyclic peptide with 17 residues in chum salmon pituitary (Kawauchi et al, 1983). It was found to be secreted from the hypothamus and stored in neurohypophysis in teleosts (Naito et al, 1985). Recently, it was found to inhibit the release of both ACTH (Baker et al, 1986) and MSH (Barber et al, 1987) indicating an ancient regulatory mechanism on ACTH and possibly POMC (or another ancient POMC) in fish that does not exist in mammals. However, in a study on trout, the distribution of POMC-derived peptides in pars

distalis and pars intermedia of teleosts was found to be similar to that of higher vertebrates (Rodrigues and Sumpter, 1983).

7.2 Grouper brain

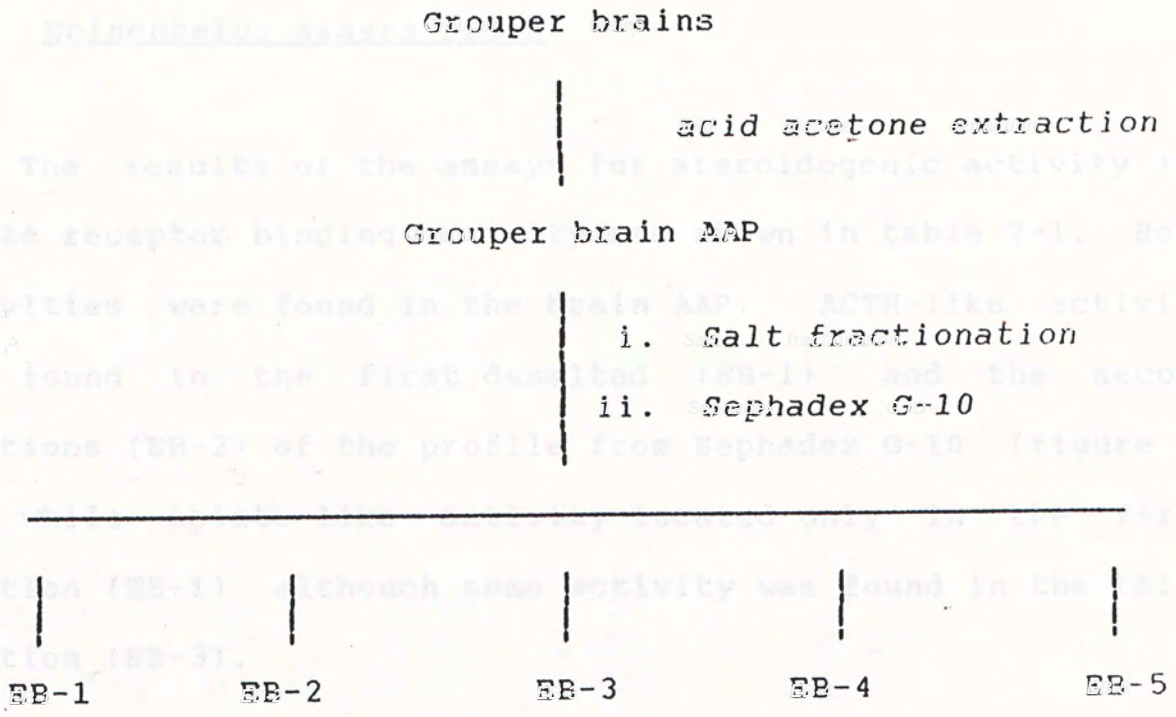
7.2.1 Introduction - *Epinephelus akaara* , the red grouper

Epinephelus akaara are small fish belonging to Class Perciformes with body length about 200 mm, living in the bottom of warm water around Korea, Japan, Taiwan and in South China Sea. Perciformes belongs to Actinopterygii which is evolved from Salmonoids during the evolution of teleosts (Young, 1983b), Salmon can be regarded as the ancester of grouper in the phylogenic point of view. Studies on this species can give information of the roles of ACTH and opiates in the adaptive evolution process of teleosts. However, there are no reports about the presence of ACTH and opiates in *Epinephelus akaara*. As a preliminary study on this species, only its brain was tested for the presence of ACTH and opiate.

7.2.2 Materials and method - extraction of grouper brain

The extraction protocol is shown in figure 7-1. The preparation of acid acetone powder (AAP) follows that of rat heart AAP (section 4.1.2.1a). The AAP was then fractionated with different concentration of NaCl as described previously (section 4.1.2.3b) and then desalted on a Sephadex G-10.

Figure 7-1 Extraction and purification of ACTH-like and opiate-like activities from grouper brain



7.2.3 Results : ACTH-like and opiate-like activities of *Epinephelus akaara* brain

The results of the assays for steroidogenic activity and opiate receptor binding activity are shown in table 7-1. Both activities were found in the brain AAP. ACTH-like activity was found in the first desalted (EB-1) and the second fractions (EB-2) of the profile from Sephadex G-10 (figure 7-2) while opiate-like activity located only in the first fraction (EB-1) although some activity was found in the third fraction (EB-3).

Table 7-1. Steroidogenic and opiate activities of *Epinephelus akaara* brain fractions eluted from Sephadex G-10

Fraction	Steroidogenic activity		Opiate receptor binding activity	
	Dose (mg)	(ng corticosterone /hr/25,000 cells)	Dose (mg)	%Binding
Control[a]	0	0.028 ± 0.008	-	-
ACTH	1 (nM)	1.511 ± 0.082 ^	-	-
"	0.33 (nM)	1.154 ± 0.065 ^	-	-
"	0.11 (nM)	0.551 ± 0.036 ^	-	-
LEK	-	-	4 (µg)	16.8 ± 1.34 ^
"	-	-	0.8 (µg)	38.1 ± 3.10 ^
"	-	-	0.16 (µg)	79.0 ± 2.76 ^^
"	-	-	0.032 (µg)	104.9 ± 2.99
EB-AAP	0.444	0.743 ± 0.060 ^	0.5	79.2 ± 17.18
"	0.049	0.501 ± 0.002 ^	0.05	114.8 ± 10.26
"	0.005	0.015 ± 0.003		
Control[b]	0	0.008 ± 0.003	-	-
ACTH	1 (nM)	0.834 ± 0.051 ^	-	-
"	0.25 (nM)	0.578 ± 0.050 ^	-	-
"	0.062 (nM)	0.130 ± 0.018 ^^	-	-
"	0.016 (nM)	0.021 ± 0.012	-	-
EB-1	1	0.241 ± 0.011 ^	0.5	7.8 ± 1.16 ^
"	0.1	0.258 ± 0.054	0.05	59.3 ± 3.47 ^
"	0.01	0.014 ± 0.007	0.005	75.7 ± 2.08 ^^
EB-2	1	0.132 ± 0.056	trace	ND
"	0.1	0.041 ± 0.010		
"	0.01	0.013 ± 0.001		
EB-3	1	0.006 ± 0.002	0.5	51.2 ± 3.30 ^
"	0.1	0.009 ± 0.004	0.05	62.0 ± 4.26 ^^
"	0.01	0.008 ± 0.003	0.005	83.2 ± 1.37 ^^
EB-4	1	0.001 ± 0.000	trace	ND
EB-5	1	0.009 ± 0.001	trace	

a: expt. 1, b: expt.2

Values represent mean ± S.E.M. of triplicate determinations.

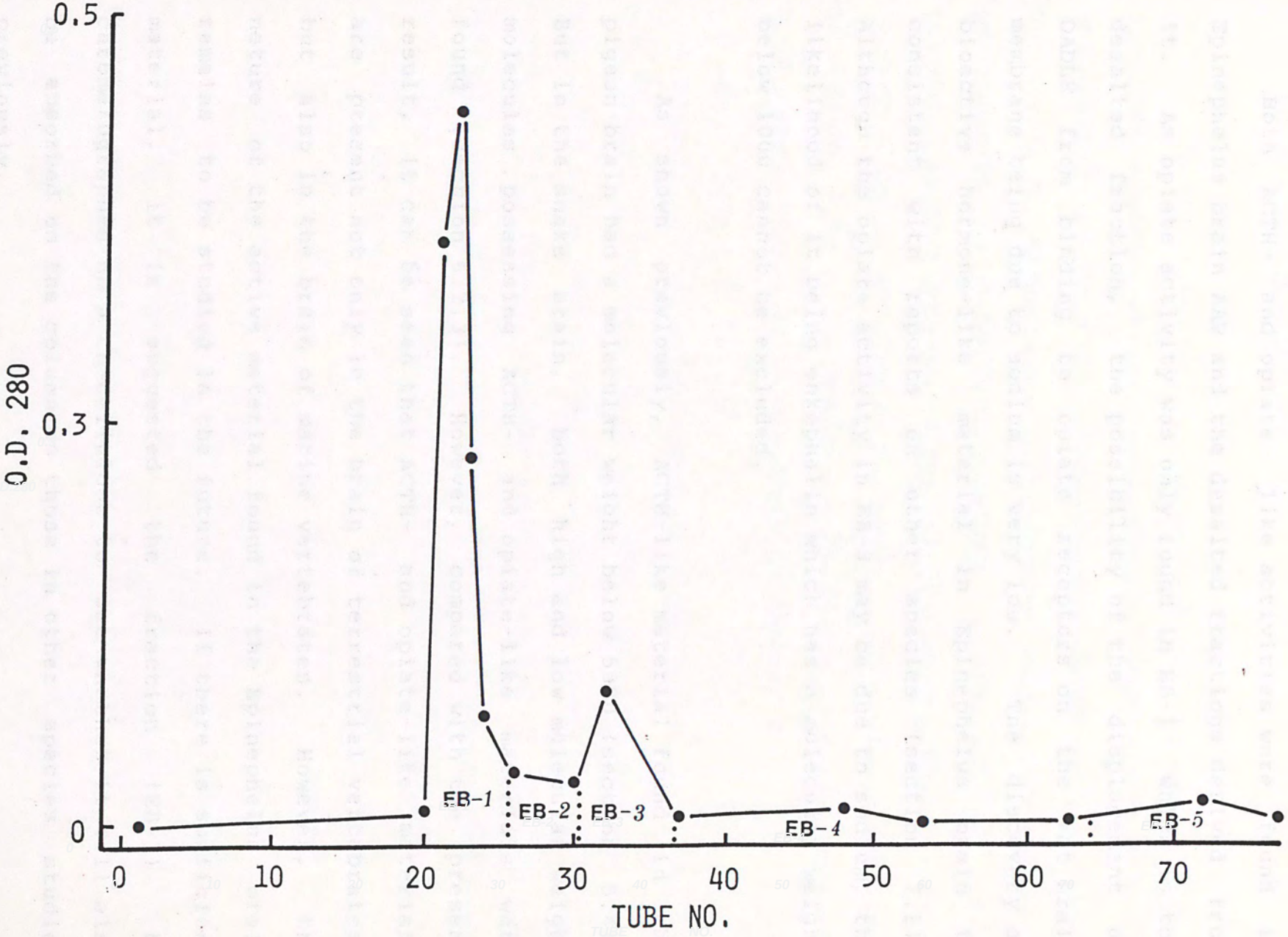
LEK : Leu-enkephalin
ND : Not determined due to insufficient material
^ : $p < 0.001$ compared with control or buffer
^^ : $p < 0.005$ compared with control or buffer

Figure 7-2

Gel filtration of fraction (200 mg) derived from grouper brain AAP after salt fractionation on Sephadex G-10 (3.5 x 80 cm). Fraction size = 5 ml.

Buffer: 0.1 M acetic acid.

Yields: EB-1, 9.3 mg; (Void volume)
EB-2, 2 mg;
EB-3, 40 mg;
EB-4, trace amounts;
EB-5, trace amounts.


0.5
0.3
0
O.D. 280
0
10
20
30
40
50
60
70
TUBE NO.
EB-1
EB-2
EB-3
EB-4
EB-5

7.2.4 Discussion - Fish Brains

Both ACTH- and opiate- like activities were found in Epinephelus brain AAP and the desalted fractions derived from it. As opiate activity was only found in EB-1 which is the desalted fraction, the possibility of the displacement of DADLE from binding to opiate receptors on the rat brain membrane being due to sodium is very low. The discovery of bioactive hormone-like material in Epinephelus brain is consistent with reports on other species (section 7.1). Although the opiate activity in EB-3 may be due to sodium, the likelihood of it being enkephalin which has a molecular weight below 1000 cannot be excluded.

As shown previously, ACTH-like material found in the pigeon brain had a molecular weight below 5000 (section 5.4). But in the snake brain, both high and low molecular weight molecules possessing ACTH- and opiate-like materials were found (section 6.2.3). However, compared with the present result, it can be seen that ACTH- and opiate-like materials are present not only in the brain of terrestrial vertebrates, but also in the brain of marine vertebrates. However, the nature of the active material found in the Epinephelus brain remains to be studied in the future. If there is sufficient material, it is suggested the fraction (EB-1) be chromatographed on a CM-cellulose to see whether it will also be absorbed on the column as those in other species studied previously.

7.3 Various tissues of flounder

7.3.1 Introduction - flounder

The winter flounder, *Pseudopleuronectes americanus*, is a flatfish belonging to Order Heterosomata and it lives near the bottom in deep sea. Heterosomata (Pleuronectes) also belongs to Actinopterygii (Young, 1983b) so flounder can also be regarded as derived from salmon. Different from the grouper, various tissues of the flounder were studied in this investigation in addition to the brain. Thus, the results can be used not only as a comparison with salmon, but also compare with lamprey that will be discussed in chapter 8.

Reports on the existence of ACTH and opiate in flounder were scarce. However, MSH, one of the peptides derived from POMC, was found in both the serum and pituitary of flounder (Baker et al, 1984). The MSH found in salmon is equal to the first 13 amino acids of the N-terminal of ACTH (Kawauchi et al, 1984). The presence of MSH in flounder thus indirectly indicates the possible existence of ACTH and POMC.

7.3.2 Materials and methods - Extraction of flounder tissues

As in the case of the extraction of snake pituitaries (section 6.3.2), two methods have been applied in the preparation of tissues of winter flounder.

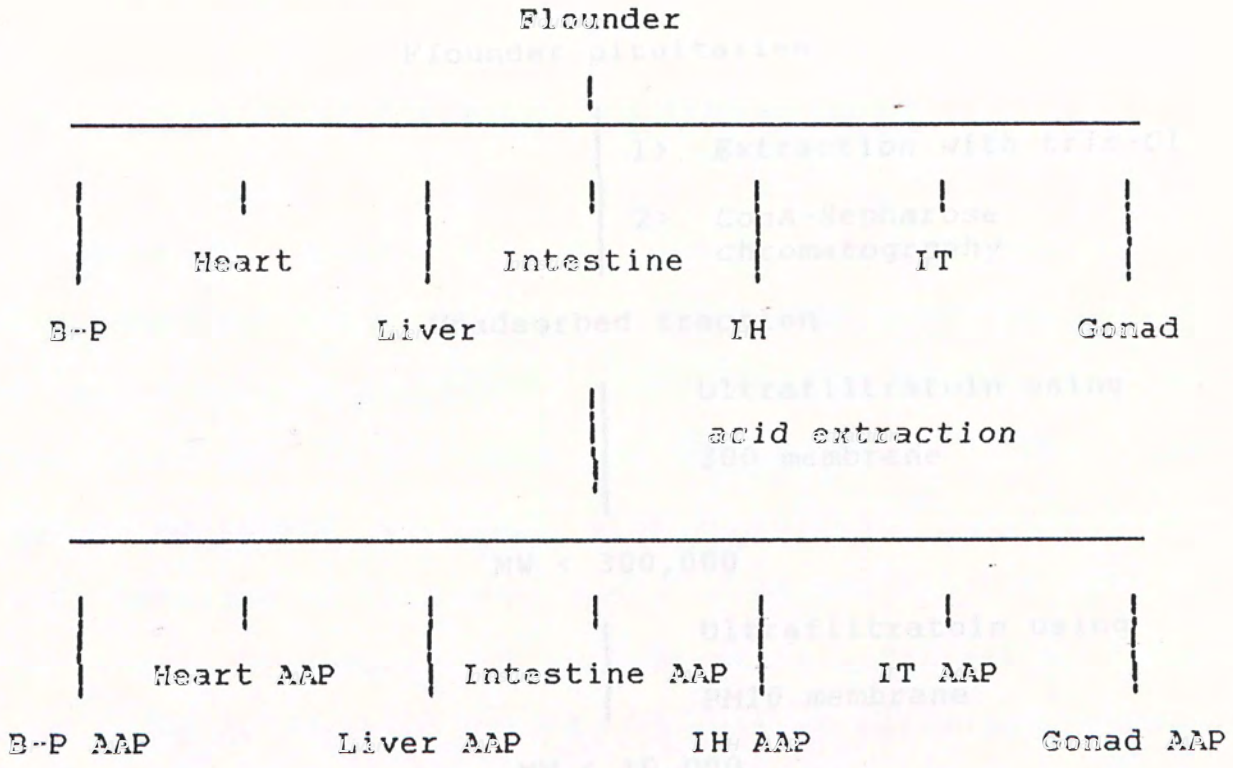
7.3.2.1 Method I

As in the case of snake pituitaries, the starting amount of flounder tissues was also little. Hence the acid extraction method used in the Method I of AAP preparation for snake pituitaries (section 6.3.2.1) was applied. However, due to small amount of samples available, no further purification is possible (figure 7-3).

7.3.2.2 Method II

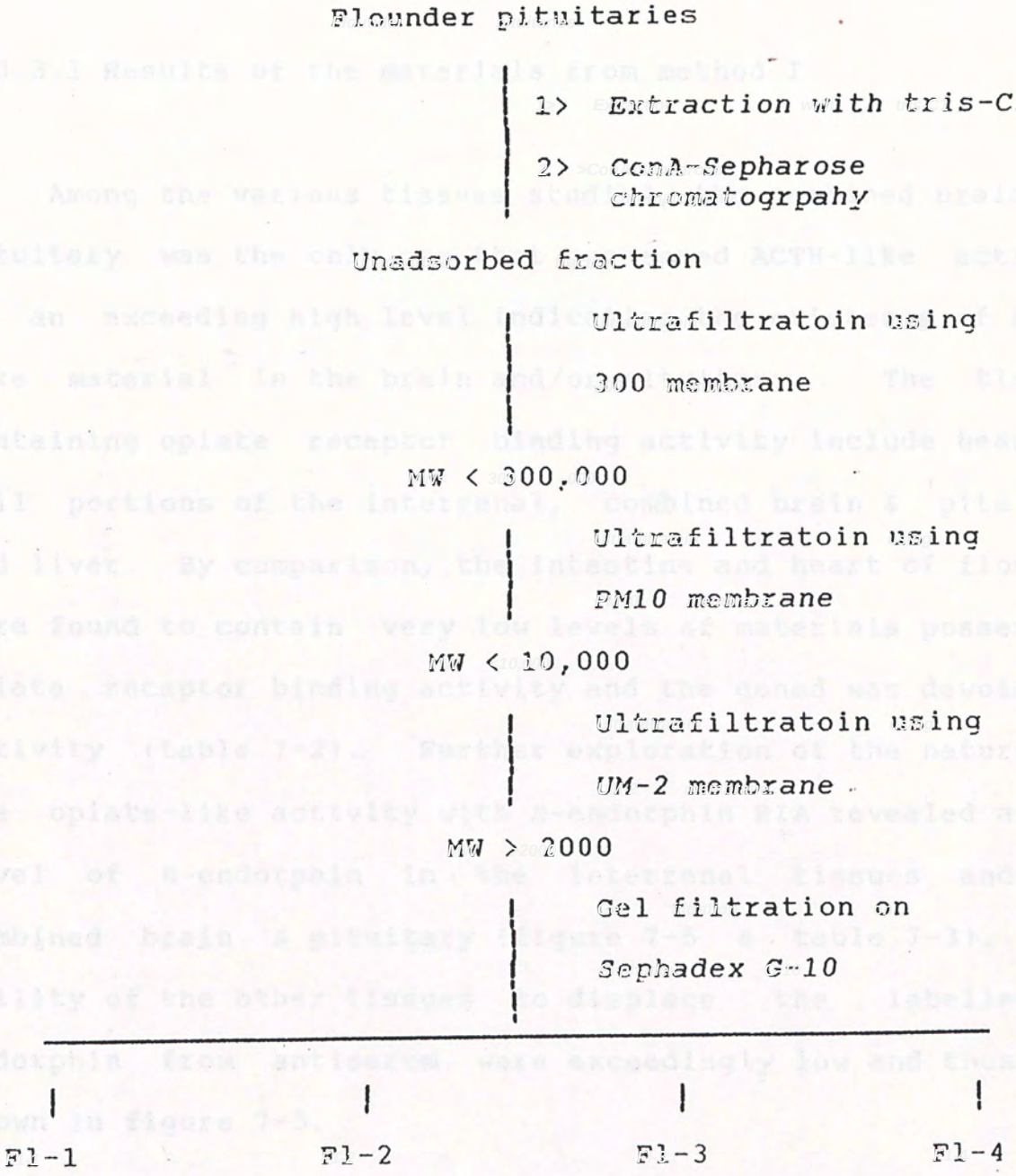
A similar procedure as in the method II of the preparation of snake pituitaries (section 6.3.2.2) was applied. The starting material was pituitaries of the same flounder, *Pseudopleuronectes americanus*. After extraction with tris-Cl buffer (pH 7.8) and affinity chromatgraphy on ConA-Sepharose, the unadsorbed fraction was concentrated through an XM-300 membrane (MW cut-off = 300,000). The filtrate (MW < 30,000) was then concentrated over a PM10 membrane (MW cut-off = 10,000) and the subsequent filtrate over a UM-2 membrane (MW cut-off = 2000), thus resulting in a retentate of a molecular weight between 10,000 and 2000. The resulting material was then desalted on a Sephadex G-10 column (figure 7-4).

Figure 7-3 Method I - Extraction of ACTH-like and opiate-like activities from various flounder tissues



B-P : Combined brain & pituitary
IH : Interrenal head
IT : Interrenal tail

Figure 7-4 Method II - Extraction and purification of ACTH-like and opiate-like activities from flounder pituitary



7.3.3 Results : ACTH-like and opiate-like activities in flounder tissues

7.3.3.1 Results of the materials from method I

Among the various tissues studied, the combined brain and pituitary was the only one that possessed ACTH-like activity at an exceeding high level indicating the existence of ACTH-like material in the brain and/or pituitary. The tissues containing opiate receptor binding activity include head and tail portions of the interrenal, combined brain & pituitary and liver. By comparison, the intestine and heart of flounder were found to contain very low levels of materials possessing opiate receptor binding activity and the gonad was devoid of activity (table 7-2). Further exploration of the nature of the opiate-like activity with β-endorphin RIA revealed a low level of β-endorphin in the interrenal tissues and the combined brain & pituitary (figure 7-5 & table 7-3). The ability of the other tissues to displace the labelled β-endorphin from antiserum were exceedingly low and thus not shown in figure 7-5.

7.3.3.2 Results of the materials from method II

Four fractions were obtained when the flounder pituitary extract was chromatographed on the Sephadex G-10 column (figure 7-6). The yield of the fourth fraction (Fl-4) was too low to permit assay for steroidogenic and opiate activities. The other fractions (Fl-1, Fl-2, Fl-3) were also found to

Table 7-2. Steroidogenic and opiate activities of various tissues of flounder AAP

AAP	Steroidogenic activity		Opiate receptor binding activity	
	Dose (mg)	(ng corticosterone /hr/25,000 cells)	Dose (mg)	%Binding
Control	0	0.006 ± 0.001	-	-
ACTH	5 (nM)	0.054 ± 0.038	-	-
"	0.25 (nM)	0.031 ± 0.017	-	-
"	0.12 (nM)	0.009 ± 0.000	-	-
LEK	-	-	3.33 (µg)	15.3 ± 1.72 ^
"	-	-	0.037 (µg)	62.8 ± 3.94 ^^
"	-	-	0.041 (µg)	92.9 ± 3.27
B-P	0.1	0.282 ± 0.012 ^	0.5	32.2 ± 1.57 ^
"	0.01	0.171 ± 0.116	0.05	87.5 ± 6.71
"			0.005	83.6 ± 2.22
Heart	0.1	0.006 ± 0.001	0.5	65.5 ± 0.06 ^
"	0.01	0.005 ± 0.000	0.05	93.8 ± 4.67
"			0.005	96.8 ± 6.03
Liver	0.1	0.005 ± 0.000	0.5	5.03 ± 2.74 ^
"	0.01	0.005 ± 0.000	0.05	88.9 ± 1.40
"			0.005	102.7 ± 3.86
Intestine	0.1	0.001 ± 0.000	0.5	88.1 ± 3.48
"	0.01	0.005 ± 0.001	0.05	111.8 ± 8.84
IH	0.1	0.006 ± 0.001	0.5	57.4 ± 0.68
"	0.01	0.006 ± 0.001	0.05	91.3 ± 3.10
"			0.005	102.4 ± 0.68
IT	0.1	0.005 ± 0.001	0.5	23.3 ± 4.98 ^
"	0.01	0.006 ± 0.000	0.05	83.8 ± 2.02 ^^
"			0.005	103.5 ± 2.27
Gonad	0.1	0.005 ± 0.000	0.5	97.7 ± 3.80
"	0.01	0.006 ± 0.000	0.05	114.6 ± 7.22

Values represent mean ± S.E.M. of triplicate determinations.

IT : Interrenal tail; IH : Interrenal head
B-P : Combined Brain & Pituitary
LEK : Leu-enkephalin
^ : $p < 0.001$ compared with control buffer
^^ : $p < 0.005$ compared with control or buffer

Figure 7-5

Displacement of [^{125}I]β-endorphin from β-endorphin antiserum by the AAP of various tissues of flounder

Labels on the figure :

1 : Interrenal head
2 : Interrenal tail
3 : Combined brain & pituitary

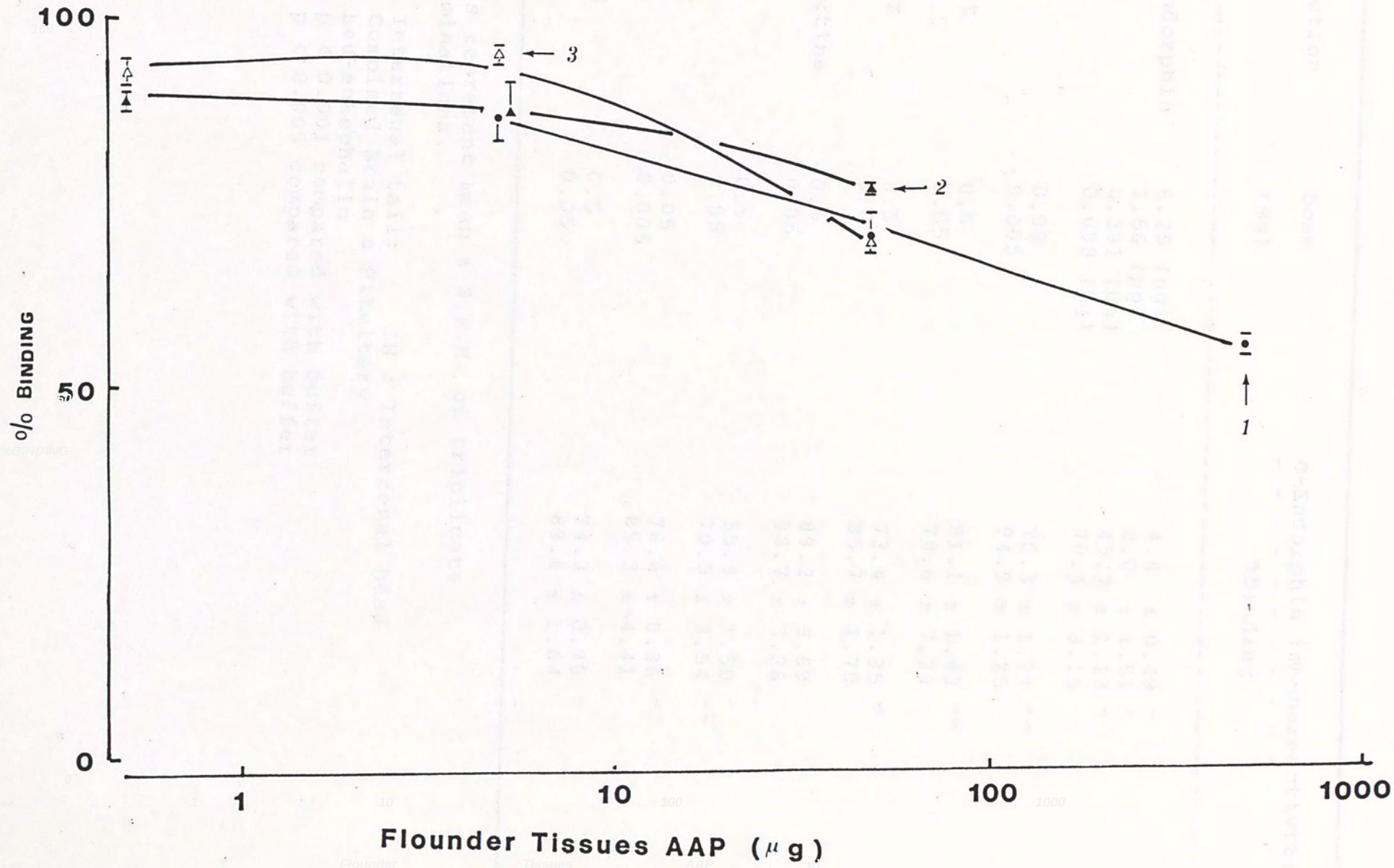

100
50
0
% Binding
3
2
1
1
10
100
1000
Flounder Tissues AAP (μg)

Table 7-3. ß-Endorphin immunoreactivity of flounder tissues AAP

Fraction	Dose (mg)	ß-Endorphin immunoreactivity %binding
ß-Endorphin	6.25 (ng)	4.4 ± 0.49 ^
"	1.56 (µg)	8.0 ± 1.51 ^
"	0.391 (µg)	45.7 ± 1.13 ^
"	0.098 (µg)	70.4 ± 3.15
B-P	0.05	70.3 ± 1.91 ^^
"	0.005	94.9 ± 1.25
Heart	0.5	81.1 ± 1.43 ^^
"	0.05	78.6 ± 7.29
Liver	0.5	73.4 ± 1.35 ^
"	0.05	85.7 ± 1.70
Intestine	0.5	89.2 ± 3.69
"	0.05	98.7 ± 3.26
IH	0.5	55.8 ± 1.50 ^
"	0.05	70.5 ± 3.54 ^^
IT	0.05	76.6 ± 0.36 ^
"	0.005	85.3 ± 4.41
Gonad	0.5	74.3 ± 0.99 ^
"	0.05	89.8 ± 1.64

Values represent mean ± S.E.M. of triplicate determinations.

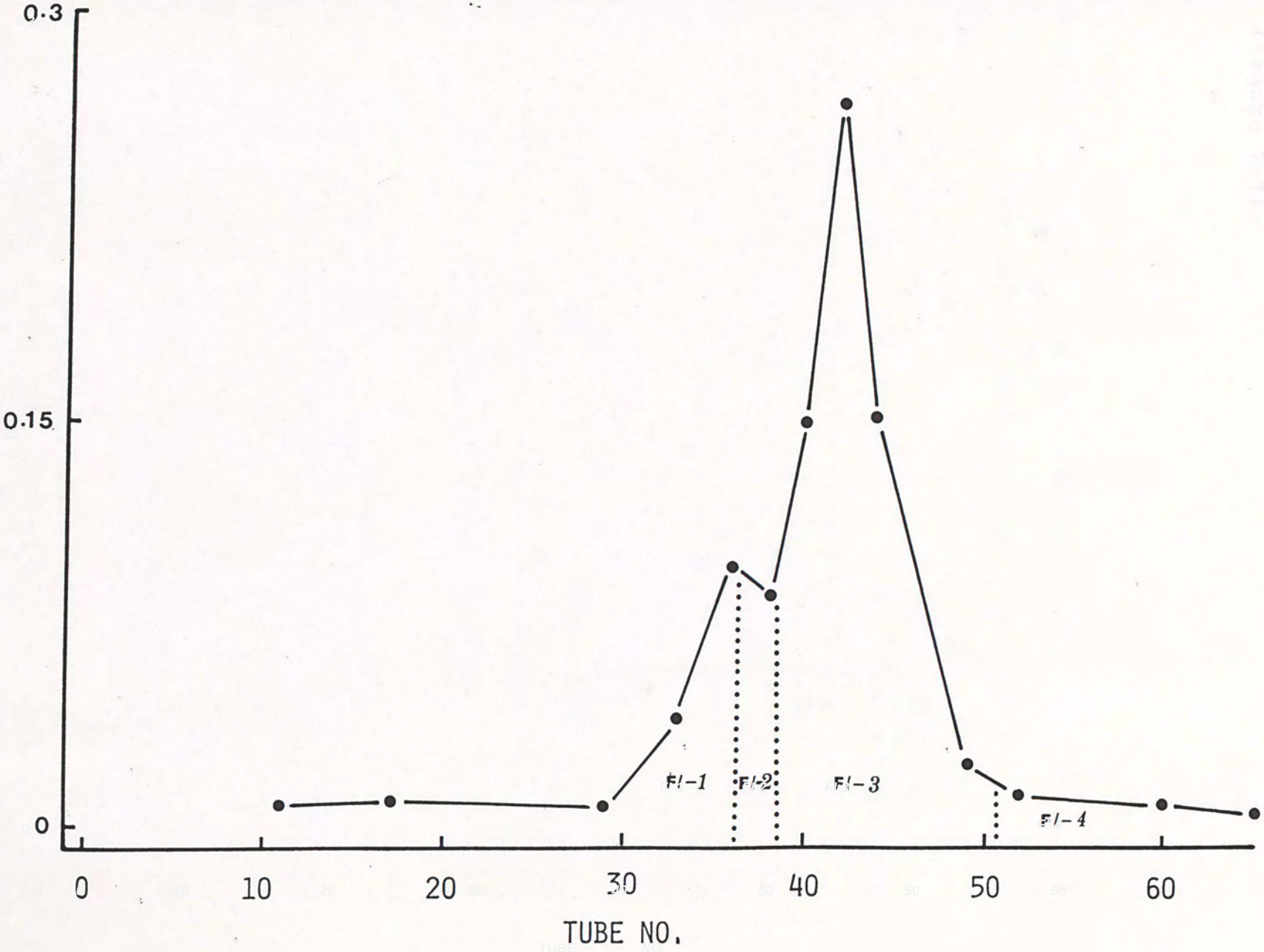
IT : Interrenal tail; IH : Interrenal head
B-P : Combined Brain & Pituitary
LEK : Leu-enkephalin
^ : $p < 0.001$ compared with buffer
^^ : $p < 0.005$ compared with buffer

Figure 7-6

Gel filtration of flounder pituitary fraction with a MW 2000-10,000 (502 mg) on a Sephadex G-10 column (3 x 83 cm). Fraction size = 5 ml.

Buffer: 0.1 M acetic acid.

Yields: Fl-1, 14.3 mg; (Void volume)
Fl-2, 155.5 mg;
Fl-3, 164.5 mg;
Fl-4, trace amount.


0.3
0.15
0
O.D. 280
Fl-1
Fl-2
Fl-3
Fl-4
0
10
20
30
40
50
60
TUBE NO.

contain opiate receptor binding activity but the ACTH-like activity was only found in the first (F1-1) desalted fraction (table 7-4). When the fractions were tested with the ß-endorphin RIA assay, the first (F1-1) desalted fraction was the only one that possessed ß-endorphin immunoreactivity (figure 7-7).

Table 7-4 Steroidogenic and opiate activities of flounder pituitary fractions eluted from Sephadex G-10

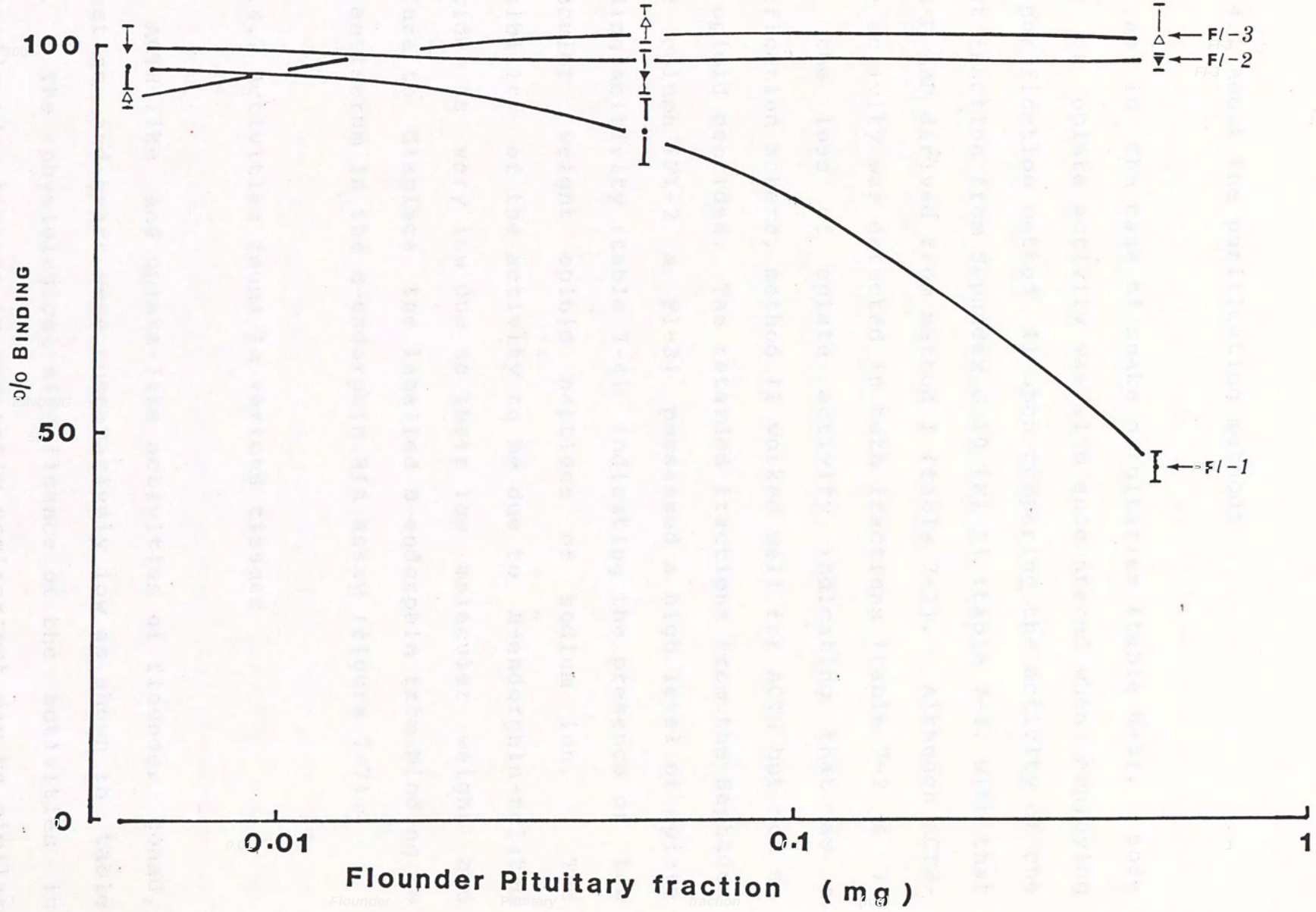
Fraction	Steroidogenic activity		Opiate receptor binding activity	
	Dose (mg)	(ng corticosterone /hr/25,000 cells)	Dose (mg)	%Binding
Control	0	0.006 ± 0.001	-	-
ACTH	5 (nM)	0.054 ± 0.038	-	-
"	0.25 (nM)	0.031 ± 0.017	-	-
"	0.12 (nM)	0.009 ± 0.000	-	-
LEK	-	-	0.313 (µg)	22.9 ± 3.14 ^
"	-	-	0.078 (µg)	52.0 ± 1.37 ^
"	-	-	0.020 (µg)	70.3 ± 0.61 ^
"	-	-	0.005 (µg)	80.4 ± 1.58 ^^
Fl-1	0.1	0.172 ± 0.029 ^^	0.5	64.4 ± 4.34 ^^
"	0.01	0.010 ± 0.001	0.05	99.3 ± 2.58
"			0.005	92.1 ± 1.37
Fl-2	1	0.005 ± 0.001	0.5	30.4 ± 0.06 ^
"	0.1	0.006 ± 0.001	0.05	66.6 ± 2.54 ^
"			0.005	100.2 ± 1.09
Fl-3	1	0.003 ± 0.000	0.5	24.3 ± 1.14 ^
"	0.1	0.006 ± 0.000	0.05	79.8 ± 0.99 ^
"			0.005	96.2 ± 2.23

Values represent mean ± S.E.M. of triplicate determinations.

LEK : Leu-enkephalin
^ : $p < 0.001$ compared with control or buffer
^^ : $p < 0.005$ compared with control or buffer

Figure 7-7

Displacement of [^{125}I]β-endorphin from β-endorphin antiserum by the fractions of flounder pituitary eluted from Sephadex G10


100
50
0
% Binding
0.01
0.1
1
Flounder Pituitary fraction (mg)
Fl-3
Fl-2
Fl-1

7.3.4 Discussion - flounder tissues

7.3.4.1 About the purification methods

As in the case of snake pituitaries (table 6-5), some loss of opiate activity was also encountered when employing the purification method II upon comparing the activity of the first fraction from Sephadex G-10 (F1-1) (table 7-4) with that of B-P AAP derived from method I (table 7-2). Although ACTH-like activity was detected in both fractions (table 7-2 & 7-4), the loss of opiate activity indicating that as a purification scheme, method II worked well for ACTH but not so for opioid peptides. The retarded fractions from the Sephadex G-10 column (F1-2 & F1-3) possessed a high level of opiate binding acitivity (table 7-4) indicating the presence of low molecular weight opioid peptides or sodium ion. The possibility of the activity to be due to β-endorphin-related peptide is very low due to their low molecular weight and failure to displace the labelled β-endorphin from binding to its antiserum in the β-endorphin RIA assay (figure 7-7).

7.3.4.2 Activities found in various tissues

ACTH-like and opiate-like activities of flounder gonad, intestine and heart were comparatively low as shown in table 7-2. The physiological significance of the activities in these flounder tissues is presently unclear but may be similar to the situation in mammals. The presence of high activities in the brain & pituitary is predictable. However, the

presence of high levels of opiate activity in the liver is not so straight-forward.

To remove the effects of possible existing proteases on the opiate receptor binding assay, the tissues were heated at 90 °C for 15 minutes before extraction with acidic medium (section 6.3.3) and furthermore, protease inhibitors such as trypsin inhibitor and bracitracin were added to the assay tube and co-incubated with the brain membrane (section 3.2.2.2) to prevent any proteolytic effect. Thus the positive result obtained from the liver seems not to be an artifact due to proteolytic activity. Tissues such as the intestine and heart only possessed low levels of opiate activity indicating that the result was not due to presence of artifacts in the medium such as sodium. The data obtained from the flounder liver may be due to an abnormally high concentration of sodium in the tissue.

However, as chromatographic purification could not be applied due to the shortage of material, no idea of the molecular size and chemical nature of the active materials was obtained. An exceedingly low level of β-endorphin immuno-reactivity was also found in flounder liver but a similar level was also reported for flounder gonad. As the former was found to possess opiate binding activity but the later was found to have none, it is reasonable to conclude that the material in flounder liver is not structurally similar to mammalian β-endorphin.

7.3.4.3 Implications of the results

As discussed previously, the interrenal was the adrenal gland of fish (section 7.1.2.2). The presence of endogenous opioid peptides such as β-endorphin in interrenal gland of fish is reminiscent of their presence in the mammalian adrenal. In fact, proenkephalin (Comb et al, 1982) and prodynorphin (Jingami et al, 1984) and their corresponding mRNAs have already been found in the mammalian adrenal medulla. The role of opiates in fish interrenals may well be similar to that in mammalian adrenals.

7.4 Eel pancreas

7.4.1 Introduction - the eel

Different from the two fishes discussed above, eel belongs to Elopomorpha and is evolved independently from salmon (Young, 1983b) in the adaptive evolution of teleosts. However, it is worth-remembering that Elopomorpha are among the most primitive of living teleosts (Young, 1983b). The existence of MSH in eel has also been reported and its level was found to change during physiological colour change of eel(Baker et al, 1984), implying a role of MSH in colour change even in primitive vertebrates like eel. Besides, the presence of met-enkephalin-like immunoreactivity was found in the gut tissue in different developmental stages of the eel recently (L'Hermite et al, 1985).

7.4.2 Materials and methods - Extraction of eel pancreas

The overall extraction protocol was shown in figure 7-8. Eel pancreas acid acetone powder (AAP) was first prepared from acetone powder (AP, Sigma) of the eel, *Electrophorus electricus*, by the method stated previously (section 4.2.2.1). The resulting AAP was then applied to Sephadex G-25 and eluted with 0.1 N acetic acid (figure 7-9).

Figure 7-8 Extraction and purification of ACTH-like and opiate-like activities from opiate-like activities from eel pancreas

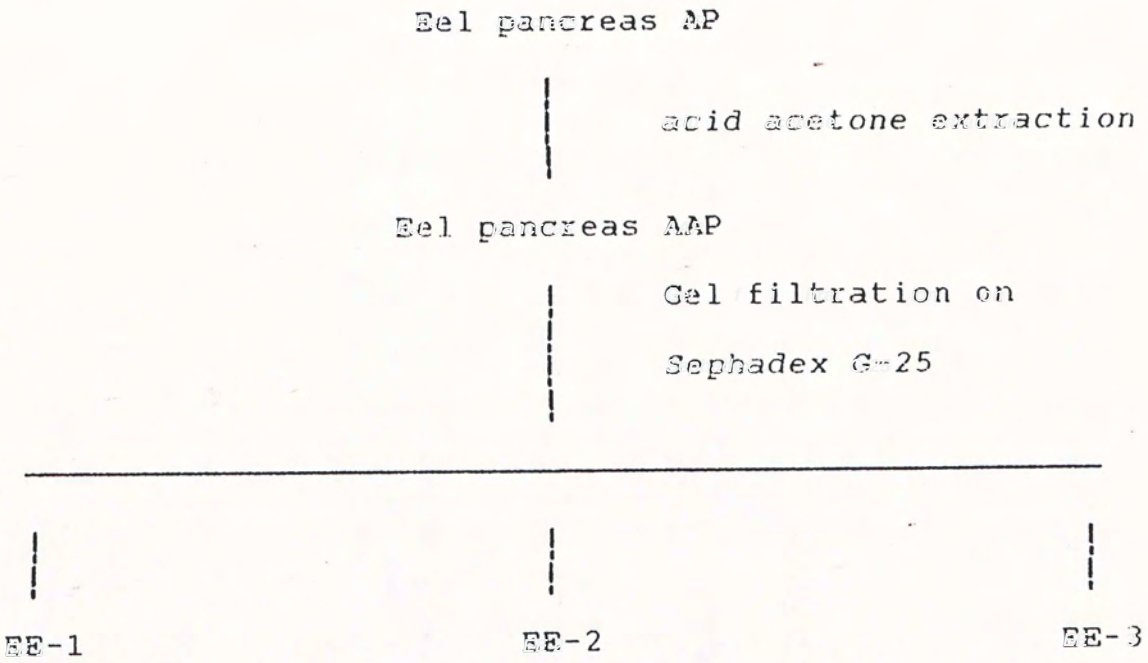


Figure 7-9

Gel filtration of eel pancreas AAP on a Sephadex G-25 column (3.5 x 80 cm). The AAP (754 mg) was dissolved in buffer, centrifuged and the resulting supernatant was them applied on a column. Fraction size = 5 ml.

Buffer: 0.1 M acetic acid.

Yields: EE-1, 180 mg; (Void volume)
EE-2, 140.4 mg;
EE-3, 2 mg;
EE-4, trace amount.

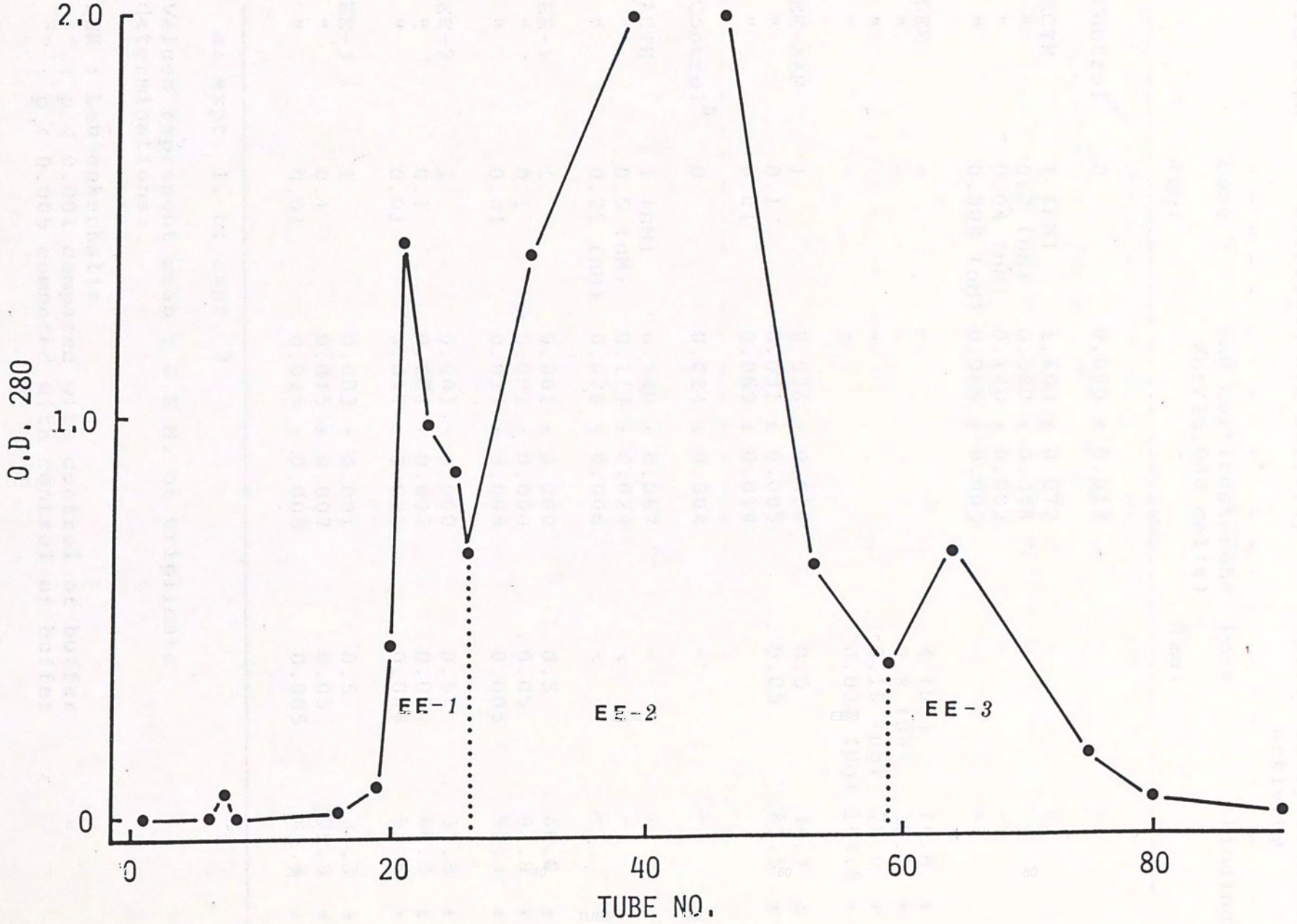

2.0
1.0
0
O.D. 280
EE-1
EE-2
EE-3
0
20
40
60
80
TUBE NO.

Table 7-5. Steroidogenic and opiate activities of eel pancreas fractions eluted from Sephadex G-25

Fraction	Steroidogenic activity		Opiate receptor binding activity	
	Dose (mg)	(ng corticosterone /hr/25,000 cells)	Dose (mg)	%Binding
Control[a]	0	0.090 ± 0.013	-	-
ACTH	1 (nM)	1.603 ± 0.072 ^	-	-
"	0.2 (nM)	0.580 ± 0.048 ^	-	-
"	0.04 (nM)	0.140 ± 0.002	-	-
"	0.008 (nM)	0.086 ± 0.005	-	-
LEK	-	-	4 (µg)	16.8 ± 1.34 ^
"	-	-	0.8 (µg)	38.1 ± 3.10 ^
"	-	-	0.16 (µg)	79.0 ± 2.76 ^^
"	-	-	0.032 (µg)	104.9 ± 2.99
EE-AAP	1	0.036 ± 0.012	0.5	34.7 ± 1.91 ^
"	0.1	0.031 ± 0.005	0.05	81.5 ± 0.64 ^
"	0.01	0.069 ± 0.014		
Control[b]	0	0.024 ± 0.004	-	-
ACTH	1 (nM)	0.540 ± 0.067 ^^	-	-
"	0.5 (nM)	0.117 ± 0.029	-	-
"	0.25 (nM)	0.029 ± 0.006	-	-
EE-1	1	0.001 ± 0.000	0.5	60.6 ± 0.24 ^
"	0.1	0.002 ± 0.000	0.05	93.8 ± 4.65
"	0.01	0.019 ± 0.005	0.005	92.3 ± 1.66
EE-2	1	0.001 ± 0.000	0.5	37.8 ± 4.22 ^
"	0.1	0.008 ± 0.002	0.05	66.9 ± 1.82 ^
"	0.01	0.017 ± 0.001	0.005	93.6 ± 1.76
EE-3	1	0.003 ± 0.001	0.5	34.3 ± 4.38 ^
"	0.1	0.015 ± 0.007	0.05	79.9 ± 2.45 ^
"	0.01	0.025 ± 0.005	0.005	83.4 ± 2.46

a: expt. 1, b: expt. 2

Values represent mean ± S.E.M. of triplicate determinations.

LEK : Leu-enkephalin
^ : $p < 0.001$ compared with control or buffer
^^ : $p < 0.005$ compared with control or buffer

7.4.3 Results : ACTH-like and opiate-like activities in eel pancreas

No steroidogenic activity was detected in any fraction of eel pancreas. Opiate receptor binding activity was found in the two low molecular weight fractions (EE-2 & EE-3) of Sephadex G-25 (table 7-5) while the opiate activity in the high molecular weight fraction (EE-1) was comparatively low.

7.4.4 Discussion - eel pancreas and mammalian pancreas

As shown in the results, low molecular weight material with opiate-like activity was found with a still undefined nature. The lower activity found in the higher molecular fraction may be due to an opiate precursor molecule and/or its fragments since no known opioid peptide has a molecular weight exceeding 5000, the molecular weight-cut off of Sephadex G-25.

The eel pancreas, unlike those of other fishes, is a compact structure surrounding the portal vein in the mid-gut. Hyperphagia and starvation markedly alter the size of the eel pancreas (Tesch and Greenwood, 1977) indicating a digestive role. Recently, eel pancreas was found to contain hormones corresponding to those of the mammalian pancreas - glucagon, insulin, and somatostatin (Naito et al, 1984). Furthermore, glucagon-like and somatostatin-like immunoreactivities were secreted from perfused eel pancreas in response to D-glucose (Ince and So, 1984), indicating structural and functional similiarities of the eel pancreas and mammals. However, ACTH was found to be generally occurred in mammalian pancreas including porcine (Alumtes et al, 1983), rat (Sanchez-Franco et al, 1981) and equine (Hon and Ng, 1986). The comparatively low level of ACTH in eel pancreas seems to be a reflection of its relatively simple structure that lacks sophisticated differentiated cells for the local synthesis of ACTH although it is capable of synthesizing other important pancreatic hormones. However, this speculation remains to be tested in the future.

DATA AND RESULT FROM CLASS AGNATHA

- Lamprey tissues

Chapter 8 DATA AND RESULT FROM CLASS AGNATHA - Lamprey tissues

8.1 Introduction - approaching the end of the tunnel

8.1.1 Lamprey is the most primitive vertebrate

The earliest vertebrates are grouped together into Agnatha (without jaws). The only living agnathan are the Cyclostomata (ring-mouthed), lampreys and hagfishes. Adult lampreys are parasites that feed on other fishes (Young, 1983c). Although the lamprey is among the simplest vertebrate, it possesses a sophisticated endocrine (Hardisty and Baker, 1982) and neuronal (Rovainen, 1982) system which regulates the overall physiological function . In order to further explore the presence of ACTH and opiates down to the simplest vertebrates as a concluding remark of this thesis, various lamprey tissues were tested.

8.1.2 ACTH and lamprey

Although the level of corticosteroids in lamprey was found to be exceedingly low (Weisbart et al, 1980), ACTH was still believed to be one of the few hormones produced from the lamprey pituitary (Hardisty and Baker, 1982). MSH- and ACTH-like activities were found at a very low level in the pituitary gland of lamprey (Eastman and Portanova, 1982; Baker and Buckingham, 1983). A recent study also indicated MSH-like immunoreactivity in the pituitary of lamprey (Dores et al,

1984) although still at a very low level. However, the failure to detect ACTH-like immunoreactivity in pars distalis and MSH-like immunoreactivity in pars intermedia of two species of lamprey by the immunocytochemical method (Nozaki and Gorbman, 1984) suggested a system of ACTH-related peptides (if there is any) in the lamprey pituitary completely different from those of higher vertebrates such as mammals (Hope and Lowry, 1981) and fishes (Rodrigues and Sumpter, 1983).

Although the presence of ACTH in lamprey is still in controversy, exogenous ACTH treatment was found to elicit a morphological response of interrenal cells (Youson, 1973) in lamprey. However, treatment with mammalian ACTH failed to show any consistent pattern of changes in plasma concentrations of various corticosteroids in adult lamprey but succeeded to do so in hagfish (Weisbart et al, 1980), the only other mordern cyclostome.

8.1.3 Endogenous opioid peptides and lamprey

Immunoreactivities of met-enkephalin (Nozaki and Gorbman, 1985), β-endorphin and γ-endorphin (Nozaki and Gorbman, 1984), which is a peptide with a sequence identical to the first 17 N-terminal amino acids of β-endorphin and having ten-fold less opioid activity (Akil et al, 1981), were found in the lamprey adrenohypophysis. However, besides the pituitary, many mammalian peptides including met-enkephalin were found in the lamprey gut (Van Noorden et al, 1977) and believed to have a paracrine function. Further, the effort of localizing of

immunoreactivities of β- and γ-endorphin in the lamprey brain was unsuccessful (Nozaki and Gorbman, 1984).

8.2 Materials and method - extraction of lamprey tissues

The specis studied was *Petromyzon marinus L.*. As the amount of starting tissues was very little, the tissues of lamprey were extracted to yield AAP with the method used for preparing snake pituitary AAP (section 6.3.2.1). Also due to the low yield after extraction, the amount of material remaining did not allow further chromagraphic purification (Figure 8-1)

8.3 Results : ACTH-like and opiate-like activities in lamprey tissues

The steroidogenic and opiate receptor binding activities of various tissues of lamprey are shown in table 8-1. The brain and liver of lamprey were found to contain considerable amount of ACTH-like materials while opiate activity was mainly located in the heart, liver, brain, gut and pituitary. No ACTH-activity was found in the heart, ovary, testis, gut and pituitary while ovary and testes did not contain assayable opiate activity. Examining the lamprey tissues in the β-endorphin RIA revealed no assayable activity in any of the tissues so the data is presented in a table (table 8-2) with the results in obtained from the highest dose tried instead of a graph.

Figure 8-1 Extraction of ACTH-like and opiate-like activities from various lamprey tissues

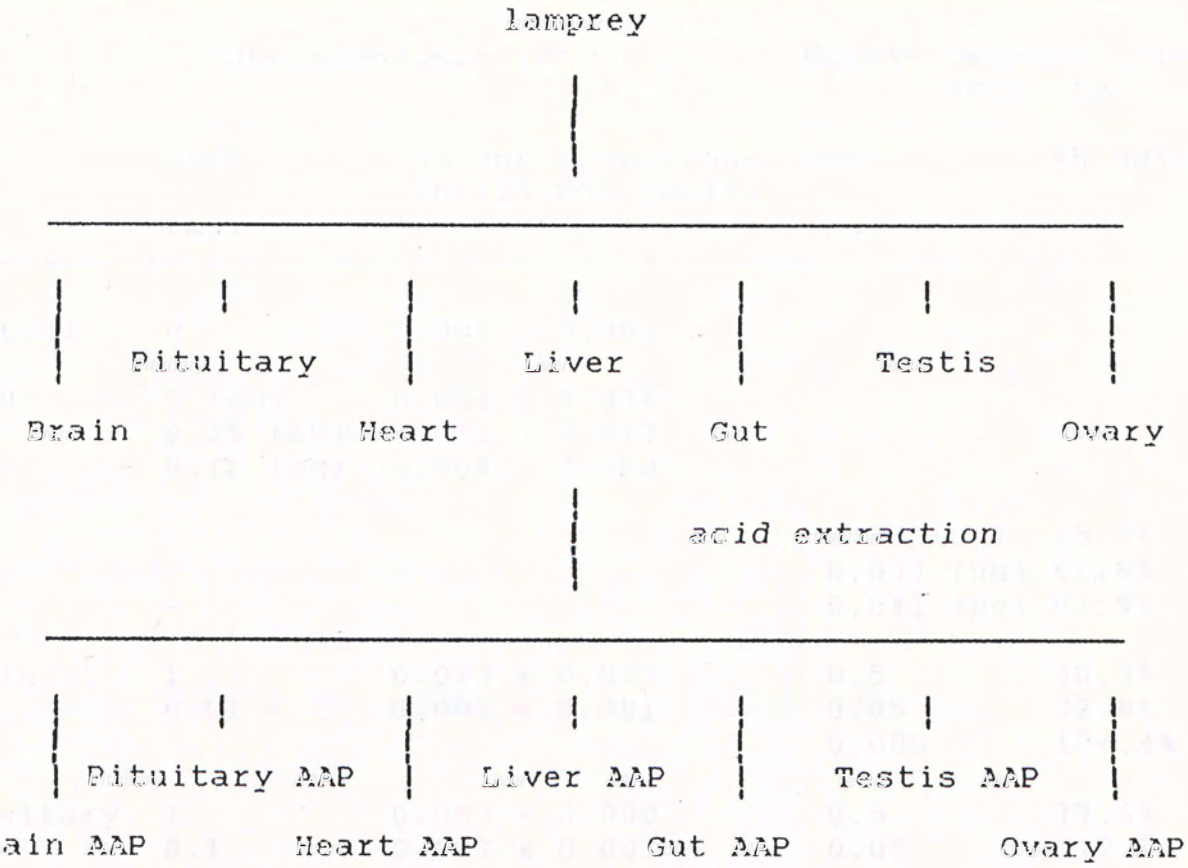


Table 8-1. Steroidogenic and opiate activities in AAPs of various lamprey tissues

AAP	Steroidogenic activity		Opiate receptor binding activity	
	Dose (mg)	(ng corticosterone /hr/25,000 cells)	Dose (mg)	%Binding
Control	0	0.006 ± 0.001	-	-
ACTH	5 (nM)	0.054 ± 0.038	-	-
"	0.25 (nM)	0.031 ± 0.017	-	-
"	0.12 (nM)	0.009 ± 0.000	-	-
LEK	-	-	3.33 (µg)	15.3% ± 1.72 ^
"	-	-	0.037 (µg)	62.8% ± 3.94 ^
"	-	-	0.041 (µg)	92.9% ± 3.27
Brain	1	0.013 ± 0.002	0.5	30.3% ± 4.24 ^
"	0.01	0.009 ± 0.001	0.05	72.8% ± 7.04
"			0.005	104.4% ± 2.16
Pituitary	1	0.003 ± 0.000	0.5	77.6% ± 2.05 ^
"	0.1	0.007 ± 0.001	0.05	112.6% ± 2.43
Heart	1	0.006 ± 0.001	0.5	27.7% ± 2.07 ^
"	0.1	0.006 ± 0.000	0.05	83.3% ± 2.76 ^
Liver	1	0.021 ± 0.001 ^	0.5	30.4% ± 2.12 ^
"	0.1	0.009 ± 0.000	0.05	72.8% ± 7.04
"			0.005	104.4% ± 2.16
Gut	1	0.001 ± 0.000	0.5	52.2% ± 1.83 ^
"	0.1	0.005 ± 0.001	0.05	100.9% ± 2.85
Testis	1	0.000 ± 0.000	0.5	93.3% ± 1.20
"	0.1	0.002 ± 0.000	0.05	105.9% ± 2.32
"			0.005	103.5% ± 2.27
Ovary	1	0.003 ± 0.001	0.5	111.8% ± 0.60
"	0.1	0.007 ± 0.000	0.05	117.9% ± 1.42

Values represent mean ± S.E.M. of triplicate determinations.

LEK : Leu-enkephalin
^ : $p < 0.001$ compared with blank
^^ : $p < 0.005$ compared with blank

Table 8-2. β-Endorphin immunoreactivity of lamprey tissues AAP

Fraction	Dose (mg)	β-Endorphin immunoreactivity %binding
β-Endorphin	6.25 (ng)	4.4 ± 0.49 ^
"	1.56 (µg)	8.0 ± 1.51 ^
"	0.391 (µg)	45.7 ± 1.13 ^
"	0.098 (µg)	70.4 ± 3.15
Brain	0.05	100.9 ± 8.10
Pituitary	0.5	121.8 ± 0.9
Heart	0.5	89.3 ± 2.71
Liver	0.5	95.7 ± 1.16
Gut	0.5	147.9 ± 0.81
Testis	0.55	119.6 ± 2.53
Ovary	0.5	81.7 ± 1.32

Values represent mean ± S.E.M. of triplicate determinations.

^ : $p < 0.001$ compared with buffer

8.4 Discussion - various lamprey tissues

8.4.1 The lamprey pituitary

8.4.1.1 ACTH-like and ß-endorphin-like activities in the lamprey and snake pituitaries

As ACTH and ß-endorphin are two well-known pituitary hormones in mammals, the most astonishing result is the absence of ACTH-like bioactivity and ß-endorphin-like immunoreactivity from the pituitary gland of lamprey. It has been reported that the levels of ACTH and MSH activities in lamprey pituitary was about 1000 fold less than those in mammals and other vertebrates (Baker and Buckingham, 1983) so the lamprey pituitary AAP was assayed for steroidogenic activity again with snake pituitary fractiions which was prepared by the same extraction method but still no assayable ACTH-like activity was found (figre 6-7) indicating the low level of activity is not due to losses during the preparation steps.

8.4.1.2 The pituitary of lamprey is different from those of others

As ACTH-like activity was found in the liver and brain but not in the pituitary of lamprey, it seems that the pituitary gland is quite different from other vertebrates. When the structure of lamprey pituitary was investigated, it

was found to be not comparable to other fishes due to the failure to identify any structural homologus in it and those of lower vertebrate fishes (Leatherland, 1975). The lmamprey Lamprey lacks a portal system and hypothalamus control of the adenohypophysis is believed to be exercised via the systemic circulation (Gorbman, 1980). However, the level of corticosteroids in lamprey is extremely low (Weisbart et al, 1980), indicating a different control system of ACTH-interrenal axis (if there is any) in lamprey.

8.4.1.3 The pituitary of lamprey and hagfish

Recent studies also concluded that the lamprey have undergone an adaptation to its parasitic mode of life and thus the cyst found inside its adenohypophysis may be a degenerated product and this adaptative process makes the characteristics found in lamprey a side branch that less resembles hagfish (Sholdice and McMillan, 1985). Hence the recent finding of ACTH activity in hagfish pituitary (Buckingham et al, 1985) does not necessarily imply that the case is also true for lamprey. Although ACTH-like bioactivity has been found by other groups (Eastman and Portanova, 1982; Baker and Buckingham, 1983; Dores et al, 1984b), no ACTH-immunoreactive cells can be identified (Nozaki and Gorbman, 1984).

8.4.1.4 Conclusion

In the present investigation, ACTH-like activity was found in the brain and liver instead of pituitary or any other

tissues, indicating that the results were not due to artifact formed during the assay or failure of the assay system itself. Furthermore, the various tissues of flounder (section 7.3) were assayed in the same assay and the presence of ACTH-like activity was detected only in the brain & pituitary tissue, supporting that the actual level of ACTH (if present) in the lamprey pituitary should be exceedingly low as predicted previously (Baker and Buckingham, 1983) and its level is probably less than previously expected.

In the case of β-endorphin, no positive result was obtained in any lamprey tissues, indicating the β-endorphin of lamprey, if present, should be at an exceedingly low level or that its structure is so different from its mammalian counterpart that it failed to displace the labelled human β-endorphin from binding to the antiserum raised against human β-endorphin (section 3.1). However, the presence of β-endorphin immunoreactivity in the flounder tissues (figure 7-5) and snake pituitary (figure 6-8) which were extracted by the same method also exclude the possibility that the β-endorphin-like activity was lost in the extraction procedure.

8.4.2 The lamprey liver

In the rat, liver possesses the lowest level of ACTH-like immunoreactivity (Saito et al, 1983) compared with other tissues. But as shown in the result (table 8-1), the reverse is true in lamprey. The high level of ACTH-like activity found in lamprey liver reflects another special feature of lamprey. The absence of measurable ACTH-like activity in the

flounder liver (table 7-2) indicates a difference of lamprey liver from those of flounder. However, the role of lamprey liver in blood sugar regulation remains in doubt. Unlike the higher vertebrates, the removal of the lamprey liver appears to have little effect on blood sugar homeostasis (Larson, 1978). It has been proposed that the kidney instead of the liver of lamprey is likely to be more important in blood sugar regulation (Murat et al, 1979) by providing a source of glucose. Furthermore, the lamprey liver is small in size (about 1% of body weight) and has a relatively small glycogen store (Plisetskaya and Kuz'mina, 1972). Unlike other vertebrates and larval (young) lamprey, adult lamprey has no gall-bladder and bile duct system (Youson, 1981).

Whether the lamprey liver reflects a primitive form of vertebrate liver or carries any special functions that differs from those in higher vertebrates remains to be studied. However, the liver of larval lamprey works more similarly to higher vertebrates than the adult lamprey liver (Youson, 1981) indicating a parasitic adaptative degeneration of the adult lamprey liver. Then it seems not too surprising that ACTH-like activity is found in lamprey liver but not in flounder liver. However, there is no idea on the possible roles of the ACTH-like activity found in the lamprey liver before the physiological function of the tissue is found.

8.4.3 Opiate activity

As in the case of flounder, the lamprey liver also contains large amounts of opiate receptor binding activity but

little β-endorphin immunoreactivity. The only method to clarify whether the opiate-like activity found in the liver of flounder and lamprey is due to artifacts is to explore its nature by further purification. If it is sodium, it will not be adsorbed on CM-cellulose or eluted in the early fractions in gel filtration. However, the presence of opiate-like activity in the brain of lamprey supports the previous finding of enkephalin receptors in lamprey brain (Fernholm et al, 1979) although the presence of the pentapeptide itself has not been reported in their results.

OVERALL SUMMARY

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Chapter 9 OVERALL SUMMARY

Chapter 9 OVERALL SUMMARY

Throughout this thesis, a phylogenetic order was followed. Although the method is systematic and easy to follow, it fails to reveal any relationship between the equivalent tissues from different species. Hence in this section the results are summarized and re-organized using a tissue-oriented approach to serve as a quick reference and overall review of the findings. However, no account has been taken of the relative potencies which will be found in the sections indicated in each table.

9.1.1 Brain

Species	Purification Method				steroidogenic activity	Opiate receptor binding activity	Section
	AAP	G-10	G-25	CMC	(No account of potency)		
	(no. of the method have been used)						
Pigeon	1		1		+	-	5.4
Snake	1		1	2	+	+ < β >	6.2.3
Gouper	1	1			+	+	7.2.3
Flounder	1	1			+	+ < β >	7.3.3
Lamprey	1				+	+	8.3

< β > : Presence of β-endorphin-like immunoreactivity

+ : Presence of activity - : absence of activity

AAP : Acid acetone powder preparation

CMC : ion exchange chromatography on CM-cellulose

G-10 : gel filtration on Sephadex G-10

G-25 : gel filtration on Sephadex G-25

9.1.2 Pituitary

Species	Purification method				steroidogenic activity	Opiate receptor binding activity	Section
	AAP	G-10	G-25	CMC	(No account of potency)		
	(no. of the method have been used)						
Snake	1		1		+	+ < β >	6.3.3
Flounder	1	1			+	+ < β >	7.3.3
Lamprey	1				-	+	8.3

< β > : Presence of β-endorphin-like immunoreactivity

+ : Presence of activity - : absence of activity

AAP : Acid acetone powder preparation

CMC : ion exchange chromatography on CM-cellulose

G-10 : gel filtration on Sephadex G-10

G-25 : gel filtration on Sephadex G-25

9.1.3 Pancreas

Species	Purification method				steroidogenic activity	Opiate receptor binding activity	Section
	AAP	G-10	G-25	CMC	(No account of potency)		
	(no. of the method have been used)						
Ovine	1	1	1	1	ND	+	4.3.3
Eel	1		1		-	+	7.4.3

+ : Presence of activity - : absence of activity

ND : Not determined

AAP : Acid acetone powder preparation

CMC : ion exchange chromatography on CM-cellulose

G-10 : gel filtration on Sephadex G-10

G-25 : gel filtration on Sephadex G-25

9.1.4 Heart

Species	Purification method			steroidogenic activity	Opiate receptor binding activity	Section
	AAP	G-10	G-25 CMC	(No account of potency)		
	(no. of the method have been used)					
Rat	1		1	+	+ < β >	4.1.3.1
Hamster	1		1	+	+ < β >	4.1.3.2
Guinea-pig	1		1	+	+ < β >	4.1.3.3
Gerbil	1		1	+	+	4.1.3.4
Turtle	1		1	-	+ < β >	6.4.3
Flounder	1	1		-	+	7.3.3
Lamprey	1			-	+	8.3

< β > : Presence of β-endorphin-like immunoreactivity

+ : Presence of activity - : absence of activity

AAP : Acid acetone powder preparation
CMC : ion exchange chromatography on CM-cellulose
G-10 : gel filtration on Sephadex G-10
G-25 : gel filtration on Sephadex G-25

9.1.5 Intestine

Species	Purification method			steroidogenic activity	Opiate receptor binding activity	Section
	AAP	G-10	G-25 CMC	*(No account of potency)*		
	(no. of the method have been used)					
Turtle	1	1	1	-	+ < β >	6.5.3
Flounder	1			-	-	7.3.3
Lamprey	1			-	-	8.3

< β > : Presence of β-endorphin-like immunoreactivity

+ : Presence of activity - : absence of activity

AAP : Acid acetone powder preparation

CMC : ion exchange chromatography on CM-cellulose

G-10 : gel filtration on Sephadex G-10

G-25 : gel filtration on Sephadex G-25

9.1.6 Gonad

Species	Purification method			steroidogenic activity	Opiate receptor binding activity	Section
	AAP	G-10	G-25 CMC	(No account of potency)		
	(no. of the method have been used)					
Bovine-testes	1		1	+	+	4.2.3
Flounder-gonad	1			-	-	7.3.3
Lamprey-testes	1			-	-	8.3
Lamprey-ovary	1			-	-	8.3

+ : Presence of activity - : absence of activity

AAP : Acid acetone powder preparation

CMC : ion exchange chromatography on CM-cellulose

G-10 : gel filtration on Sephadex G-10

G-25 : gel filtration on Sephadex G-25

9.1.7 Other tissues

Species	Purification method				steroidogenic activity	Opiate receptor binding activity		Section
	AAP	G-10	G-25	CMC	(No account of potency)			
	(no. of the method have been used)							
Flounder-liver	1				-	+		4.2.3
Flounder-interrenal	1				-	+	< β >	8.3
Lamprey-liver	1				+	+		8.3

< β > : Presence of β-endorphin-like immunoreactivity

\+ : Presence of activity - : absence of activity

AAP : Acid acetone powder preparation

CMC : ion exchange chromatography on CM-cellulose

G-10 : gel filtration on Sephadex G-10

G-25 : gel filtration on Sephadex G-25

GENERAL DISCUSSION

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Chapter 10 GENERAL DISCUSSION

10.1 From the definitions to the methods

In the last chapter, the results of eight different tissues from thirteen different species (six of them being mammalian species) have been discussed. After all the results have been presented, it is time to give a general discussion on the studies described in this thesis. However, writing a general discussion on so many results is actually a more difficult task than writing a combination of all the previous discussions in each chapter. To avoid becoming lost in the complicated data, this chapter starts from the very basic item - the definitions.

The objective of this study was to investigate the existence of ACTH-like and opiate-like activities in various vertebrates. The term, ACTH-like activity, was defined as the ability to stimulate dispersed rat adrenal decapsular cells to produce corticosterone (section 3.1). β-Endorphin-like immunoreactivity was defined as the ability to displace ^{125}I-β-endorphin from binding to a specific antiserum raised in rabbit (section 3.3). On the other hand, the more general opiate-like activity was defined as the ability to displace DADLE from binding to opiate receptors located in the rat brain synaptosomal fraction (section 3.2). Immediately, it can be seen that these definitions are limited by the assay methods used in this thesis. If other assay methods had been

applied, the definitions would have been different.

Regarding the steroidogenesis bioassay and the opiate receptor binding assay, the most serious drawback of these definitions is that we have been defining the activities found in tissues of various species using the response in rat tissues as a parameter for their measurement. Studies on different species revealed a much greater structural variety of protein between vertebrates in different classes than those in the same class in the phylogenetic tree (Matsuda 1982). The problem was more serious when the tissues of lower vertebrates were studied. At this point in time the actual structures of, say, the ACTH and ß-endorphin of lamprey are unknown. What endogenous opiate might occur in the pigeon brain is also a question. Maybe these molecules are active in their own species but are not so in rat tissues and thus they were not detected by these two methods in this investigation.

The application of another method, say, the RIA of ß-endorphin, may or may not solve this problem or perhaps the problem may be even worse. The use of antibodies against mammalian ß-endorphin to search for ß-endorphin-like immunoreactivity in lower vertebrates is a sensitive method but great care should be exercised in interpreting the results. The discovery of endorphins structurally and immunologically different from mammalian ß-endorphin in the salmon pituitary (Takahashietal, 1984) is a good example illustrating the risk of using ß-endorphin RIA to demonstrate the presence of these molecules in lower vertebrates. Furthermore, as the structures of ß-endorphins of lower vertebrates were different

from those of mammalian species, a negative result in the β-endorphin RIA does not exclude the possible existence of β-endorphin in the tissue of lower vertebrates studied.

As no 'perfect' definitions on the activities found in the tissues can be established due to the limitations of the detection methods used, the limitations of the definitions used must be borne in mind and great care must be taken to avoid jumping to false conclusions.

10.2 From the methods to the results

After the results have been calculated from the data, the most important question to answer is what is implied in the results. This highly depends on the sensitivity and specificity of the assay methods and the efficiency of the purification methods. The use of assay systems and purification methods should be compromised since the more specific the assay method, the narrower the range of the material being looked for and thus the chance of missing a structurally or functionally similar material will be increased. This is why the opiate receptor binding assay is a better choice than a highly specific RIA for enkephalin, β-endorphin or any other endogenous opioid peptides especially in a preliminary scan for opiate activities.

However, β-endorphin RIA is still a valuable tool as a complementary assay system for both the ACTH and receptor binding assay since much more information could be obtained from the combined results than the individual findings. For example, positive results in both the β-endorphin RIA and steroidogenic bioassay imply a possible local synthesizing system for the two hormones since ACTH and β-endorphin are derived from the same precursor in higher vertebrates. On the other hand, positive results in both β-endorphin RIA and opiate receptor binding assay provide strong evidence for the presence of an opiate-like material that is structurally similar to β-endorphin and is able to bind to opiate receptor

specifically.

However, jumping to the conclusion that the materials with positive results in the assay systems reflect the presence of the hormones themselves is very dangerous. For example, a putative β-endorphin precursor was identified from human placenta by both receptor binding assays and RIA (Houck et al, 1980) but the substance was suspected to be an IgG (Julliard et al, 1980) having a constant chain with a sequence similar to that of β-endorphin. These controversial results give a serious warning against possible misinterpretation of the results. However, the methods were still applied because they are relatively simple, fast, sensitive and also possess a relatively high degree of specificity (chapter 3).

As specific binding does not necessarily imply bioactivity, a bioassay of opiate activity (such as the hot plate test described in chapter 5) is really a good choice as a complementary assay for opiate receptor binding assay and the β-endorphin RIA. Furthermore, it is also a good assay method when the sample has been purified to a certain degree and enough purified material is available. The problem of using bioassay is far more complex than opiate receptor binding assay and β-endorphin RIA. It cannot avoid the pitfall of using mammalian tissues for the testing of activitiers in that of lower vertebrates. Furthermore some opioids may be agonists in some tissues and antagonists in others and so the choice of tissue used in the bioassay of opiates depends on the opioid peptides of interest. For example, hamster vas deferens has only δ receptors while

rabbit vas deferens has k receptors. To test μ receptor-specific opiates, the rat vas deferens is a better choice (Kosterlitz et al, 1985). The specificities of different opioid peptides for binding to different receptors was discussed in section 3.2.3.2.

Additional information can be obtained from chromatographic behaviours of the materials. In this investigation, the presence of both high and low molecular weight materials may or may not occur depending on the species and the type of tissues studied. As a reference, the molecular weights of relevant peptides are shown in table 6-6. Furthermore, most of the active materials were found to be adsorbed on the CM-cellulose column. Although an accurate examination of the characteristic of the materials that possess ACTH and/or opiates such as β-endorphin has not been carried out, it is pertinent to point out that mammalian ACTH and β-endorphin are known to be retarded on CM-cellulose (Ng et al, 1981), indicating a similar behavior of these materials to mammalian ACTH and β-endorphin.

10.3 From the results to their implications

The results can be discussed in two completely different ways: using a species-oriented approach or a tissue-oriented approach. The results are presented using a species-oriented approach in this thesis (chapter 4-8) while they are summarized with a tissue-oriented approach in chapter 9.

10.3.1 ACTH-like and opiate-like materials in different vertebrates

Examining the experimental results at the direction parallel to the phylogenetic tree, the only vertebrate class that has not been studied in this thesis is the amphibian. This ignorance is due to the lack of access to a sizeable amount of amphibian tissues. Actually, a study on the amphibians could be very challenging since it links the evolution of vertebrates from fishes to reptiles. To fill the gap, a quick review of the findings on ACTH and opiates in amphibians is presented here:

ACTH-like materials were found in the frog pituitary and hypothalamus (Jegou et al, 1983; Campantico et al, 1985). Dynorphin was also reported in toad central nervous system (Cone and Goldstein, 1982). β-Endorphin has been reported in bullfrog (Yui, 1983) and frog (Jackson et al, 1980; Jegou et al, 1983) tissues. Studies on the toad indicated a proenkephalin-like precursor in the brain (Kilpatrick et al,

1983) which is structurally related to that of mammals but is devoid of leu-enkephalin sequences (Martens and Herbert, 1984) indicating that leu-enkephalin is a 'younger' opioid peptide compared with met-enkephalin in the vertebrate evolution.

Then combining the above results with what has been found in the previous chapters, it seems that ACTH and opiates are universally distributed in various vertebrates indicating that these peptides have an old evolutionary age in the vertebrate system. It has been proposed that hormones and other intercellular messenger molecules of vertebrates had their evolutionary origins in the microbes. When the highly complex system of vertebrates were developed, these molecules were adopted by the internal regulatory systems such as nervous and endocrine systems (Roth et al, 1986). Pituitary, the master gland of the endocrine system, is a special structure unique to the vertebrates (Holmes and Ball, 1974b). The observation that ACTH-like bioactivity and ß-endorphin-like immunoreactivity were exceedingly low in the pituitary of the lamprey (chapter 8), the most primitive vertebrate, seems to give some hints to the evolutionary age when ACTH and ß-endorphin were adopted into the pituitary gland and acted as important pituitary hormones of the higher vertebrates. However, we cannot exclude the possibility that the ACTH-like materials in lamprey possess little or no activity in mammalian adrenal cells and thus did not elicit a biological response in rat adrenal decapsular cells; the ß-endorphin-like materials in lamprey possess insufficient structural similiarity to mammalian ß-endorphin and thus failed to displace the mammalian ß-endorphin from binding to the

antibody.

Nevertheless, the results seem to suggest that ACTH and β-endorphin do not play a role as important pituitary hormones in the lamprey. Whether this is due to an adaptive degeneration or an incomplete evolution of the pituitary gland in the lamprey remains to be studied in the future.

10.3.2 ACTH-like and opiate-like materials in different tissues

From another point of view, we can cut cross sections from the phylogenetic tree and give a look at the results obtained from various tissues as shown in chapter 9. The discovery of the activities of these hormones in extra-pituitary tissues has a different meaning from that discussed in the last section. In the case of the extra-pituitary tissues of the amphibian, β-endorphin-like and enkephalin-like activities have been reported in the frog retina and brain (Jackson et al, 1980).

The results, as summarized in chapter 9, indicate the wide distribution of the hormones in various tissues. Their possible local functions have been discussed in the individual chapters so they will not be discussed here again. However, a general impression formed after examining the results in chapter 9 is that there is a trend of decreasing chance of finding the activities of these hormones in extra-pituitary tissues when lower vertebrates were studied. Although the accuracy of this impression has not been examined, it raises a

question whether the importance of these extra-pituitary hormones is increasing in the evolutionary process in the vertebrates or just indicates the structural variation of these peptides in lower vertebrates from that in higher vertebrates. The question can be answered only when these materials has been purified and characterized.

10.4 From the implications to the future perspectives

As a concluding remark of this thesis, the trends for further studies based on the implications of the results is discussed. The implications of the results, as stated above, can be summarized as: First, ACTH and ß-endorphin seem to become important pituitary hormones at an early stage of vertebrate evolution (section 10.3.1). Second, ACTH and ß-endorphin are widely distributed in various tissues of different vertebrates. Their levels in these extra-pituitary tissues seems to increase from lower to higher vertebrates (chapter 9) and thus implying a possible increase in physiological significance which may relate to the process of evolution in the molecular level (section 10.3.2).

The first thing to do before one can continue the research is to test the validity of the two hypothesis mentioned above by further clarifying the actual structures and properties of these peptides in lower vertebrates to see whether the results are due to the limitations of the assay methods (i.e. the limitations of the definitions). If the limitations of the methods are proved to be not so important, subsequent research will be very exciting and interesting. The ACTH and ß-endorphin may play a very important role in the adaptative process of vertebrate evolution. Further studies to explore their local functions in the periperal tissues in vertebrates would be worthwhile. Then we will probably arrive at the question of how the ACTH and ß-endorphin (i.e. POMC,

their precursor) win the natural selection (Darwin, 1979) game at the molecular level in the vertebrate life.

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